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SEARCH REQUEST FORM

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Please provide a detailed statement of the search topic, and describe as specifically as possible the subject matter to be searched. Include the elected species or structures, keywords, synonyms, acronyms, and registry numbers, and combine with the concept or utility of the invention. Define any terms that may have a special meaning. Give examples or relevant citations, authors, etc, if known. Please attach a copy of the cover sheet, pertinent claims, and abstract.

Title of Invention: _____

Inventors (please provide full names): _____

_____

Earliest Priority Filing Date: _____

***For Sequence Searches Only** Please include all pertinent information (parent, child, divisional, or issued patent numbers) along with the appropriate serial number.*

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FILE LAST UPDATED: 1 Apr 2003 (20030401/ED)

This file contains CAS Registry Numbers for easy and accurate substance identification.

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L89 ANSWER 1 OF 5 HCAPLUS COPYRIGHT 2003 ACS
AN 2002:869478 HCAPLUS
DN 137:348811
TI Staining agents and protocols for characterization of malignant cells in culture
IN Naus, Gregory J.; **Kornblith, Paul L.**; Burholt, Dennis R.; Meyer, Michael P.
PA USA
SO U.S. Pat. Appl. Publ., 8 pp.
CODEN: USXXCO
DT Patent
LA English
IC ICM G01N033-53
ICS C07K016-00
NCL 435007100
CC **9-10** (**Biochemical Methods**)
Section cross-reference(s): 1, 8, 15
FAN.CNT 3

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	US 2002168679	A1	20021114	US 1998-95993	19980611
	US 2001051353	A1	20011213	US 1998-189310	19981110
	US 6416967	B2	20020709		
PRAI	US 1996-679056	A2	19960712		
	US 1998-39957	A2	19980316		
	US 1998-95993	A2	19980611		

AB An improved system for screening a multiple of candidate therapeutic or chemotherapeutic agents for efficacy as to a specific patient, in which a tissue sample from the patient is harvested, cultured and sep. exposed to a plurality of treatments and/or therapeutic agents for the purpose of objectively identifying the best treatment or agent for the particular patient. Specific method innovations such as tissue sample prepn. techniques render this method practically as well as theor. useful. The identity of the malignant cells in culture is advantageously confirmed using binding reagents/staining systems specific for epithelial cells, since carcinomas are ubiquitously epithelial in nature. Cells of interest and thus confirmed as epithelial/carcinomal may then be assayed for

sensitivity to an infinite variety of malignancy treating agents including chemotherapeutic agents, radiation, immunotherapy, and so on.

ST malignant human carcinoma epithelial cell culture staining agent binder; carcinomal epithelial cell system chemotherapeutic antitumor radiation immunotherapy sensitivity

IT Keratins
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(AE1 and AE3; staining agents and protocols for characterization of malignant cells in culture)

IT Antibodies
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(CAM; staining agents and protocols for characterization of malignant cells in culture)

IT Antibodies
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(anti-cytokeratin; staining agents and protocols for characterization of malignant cells in culture)

IT Antibodies
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(anti-epithelial-membrane-antigen; staining agents and protocols for characterization of malignant cells in culture)

IT Cell
(cancer; staining agents and protocols for characterization of malignant cells in culture)

IT Mucins
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(gene MUC1; staining agents and protocols for characterization of malignant cells in culture)

IT **Antitumor agents**
Chemotherapy
Immunotherapy
Radiotherapy
(improved system for screening of; staining agents and protocols for characterization of malignant cells in culture)

IT Antibodies
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(monoclonal, AE1 cytokeratin-specific; staining agents and protocols for characterization of malignant cells in culture)

IT Antibodies
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(monoclonal, AE1/AE3 cytokeratin-specific; staining agents and protocols for characterization of malignant cells in culture)

IT Antibodies
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(monoclonal, AE3 cytokeratin-specific; staining agents and protocols for characterization of malignant cells in culture)

IT Antibodies
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(monoclonal, epithelial membrane antigen-specific; staining agents and protocols for characterization of malignant cells in culture)

IT **Animal tissue culture**
Binders
Drug screening
Epithelium
Human
Staining, biological

Transformation, neoplastic
(staining agents and protocols for characterization of malignant cells in culture)

IT 9003-99-0D, Peroxidase, conjugate with streptavidin 9013-20-1D, Streptavidin, conjugate with peroxidase 78849-24-8, Chromagen
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(staining agents and protocols for characterization of malignant cells in culture)

L89 ANSWER 2 OF 5 HCAPLUS COPYRIGHT 2003 ACS
AN 2002:315130 HCAPLUS
DN 136:306444
TI Method for tandem genomic/proteomic analyses of proliferating cells
IN **Kornblith, Paul L.**; Donovan-Peluso, Maryann
PA **Precision Therapeutics**, Inc., USA
SO PCT Int. Appl., 63 pp.
CODEN: PIXXD2
DT Patent
LA English
IC ICM C12Q
CC **9-16** (**Biochemical Methods**)
Section cross-reference(s): 1, 3, 14
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI WO 2002033117	A2	20020425	WO 2001-US32540	20011018
WO 2002033117	A3	20020627		

W: AE, AG, AL, AM, AT, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, CZ, DE, DE, DK, DK, DM, DZ, EC, EE, EE, ES, FI, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU
RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG

PRAI US 2000-691492 A 20001018
US 2001-273281P P 20010302

AB The invention concerns a novel process for producing data characterizing nucleic acids in proliferating cells using a tandem protocol for simultaneously building and expanding genomics/proteomics cancer databases while coordinating present and future genomic/proteomic individual patient assays by collecting a tissue sample including the proliferating cells; mech. dividing the sample into cohesive multicellular particulates, and growing and analyzing the resulting cells. Sets of genetic data can be analyzed along with sets of corresponding clin. data, including phenotypic data, to form profiles that can aid in identifying proliferative diseases and in prognoses. A method for diagnosing proliferative diseases using the described assay methods is also provided. Lastly, a computing device is provided which permits searching anal. and entry of the genetic data, the corresponding clin. data and/or profiles.

ST genome proteome cancer database diagnosis nucleic acid DNA RNA

IT Gene, animal
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(HSV-TK; method for tandem genomic/proteomic analyses of proliferating cells)

IT Mammary gland
Ovary, neoplasm
(carcinoma; method for tandem genomic/proteomic analyses of proliferating cells)

IT Neuroglia

(glioblastoma; method for tandem genomic/proteomic analyses of proliferating cells)

IT Alleles
Animal cell
Animal tissue
Animal tissue culture
Apoptosis
Brain, neoplasm
Cell proliferation
Chemotherapy
Computers
Cryopreservation
DNA microarray technology
Databases
Diagnosis
Drug screening
Fluorescent substances
Gene therapy
Genetic polymorphism
Genome
Human
Immunotherapy
Luminescence, bioluminescence
Neoplasm
Phenotypes
Process automation
Prognosis
Radiotherapy
Staining, biological
(method for tandem genomic/proteomic analyses of proliferating cells)

IT DNA
Nucleic acids
RNA
RL: ANT (Analyte); DGN (Diagnostic use); PRP (Properties); ANST (Analytical study); BIOL (Biological study); USES (Uses)
(method for tandem genomic/proteomic analyses of proliferating cells)

IT Probes (nucleic acid)
RL: ARG (Analytical reagent use); ANST (Analytical study); USES (Uses)
(method for tandem genomic/proteomic analyses of proliferating cells)

IT Reagents
RL: ARU (Analytical role, unclassified); ANST (Analytical study)
(method for tandem genomic/proteomic analyses of proliferating cells)

IT Gene, animal
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(method for tandem genomic/proteomic analyses of proliferating cells)

IT Proteome
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(method for tandem genomic/proteomic analyses of proliferating cells)

IT Hormones, animal, biological studies
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(method for tandem genomic/proteomic analyses of proliferating cells)

IT **Lymphocyte**
(natural killer cell; method for tandem genomic/proteomic analyses of proliferating cells)

IT Disease, animal
(proliferative; method for tandem genomic/proteomic analyses of proliferating cells)

IT Kidney, neoplasm
(renal cell carcinoma; method for tandem genomic/proteomic analyses of proliferating cells)

IT 56-65-5, ATP, uses 56-81-5, Glycerol, uses 67-68-5, DMSO, uses 69-65-8, Mannitol
RL: NUU (Other use, unclassified); USES (Uses)

(method for tandem genomic/proteomic analyses of proliferating cells)
IT 7727-37-9, Nitrogen, uses
RL: NUU (Other use, unclassified); PRP (Properties); USES (Uses)
(method for tandem genomic/proteomic analyses of proliferating cells)
IT 82410-32-0, Ganciclovir
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(method for tandem genomic/proteomic analyses of proliferating cells)

L89 ANSWER 3 OF 5 HCAPLUS COPYRIGHT 2003 ACS
AN 2001:906155 HCAPLUS
DN 136:17691
TI Method for using multicellular particulates to analyze malignant or hyperproliferative tissue
IN **Kornblith, Paul L.**
PA USA
SO U.S. Pat. Appl. Publ., 24 pp., Cont.-in-part of U. S. 5,728,541.
CODEN: USXXCO
DT Patent
LA English
IC ICM C12Q001-02
ICS C12Q001-24; C12Q001-18
NCL 435029000
CC **9-4** (**Biochemical Methods**)
Section cross-reference(s): 1, 14
FAN.CNT 3

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	US 2001051353	A1	20011213	US 1998-189310	19981110
	US 6416967	B2	20020709		
	US 5728541	A	19980317	US 1996-679056	19960712
	US 2002168679	A1	20021114	US 1998-95993	19980611
	US 2002192638	A1	20021219	US 2002-205887	20020726
PRAI	US 1996-679056	A2	19960712		
	US 1998-39957	A2	19980316		
	US 1998-95993	A2	19980611		
	US 1998-40161	A1	19980317		

AB The invention concerns a comprehensive and integrated system for monitoring (identifying, tracking and analyzing) an individual patient's malignancy through the duration of a malignancy as to a specific patient is provided. The method of the present invention allows for initial identification of a malignancy, identification of malignancy-specific cellular or secretory markers, identification of cellular or secreted markers indicative of complications, study of the invasiveness and aggressiveness of the malignancy, study of the growth rate of the malignancy, study of the effect of therapies on the malignancy as compared to control cells of the same patient (chemo-sensitivity vs. toxicity) and the identification of a therapeutic index (i.e., the ratio of chemo-sensitivity:toxicity), study of tumor morphol. and study of histol., cytochem. and immunocytochem. markers.
ST tumor malignancy therapy immunoassay histol cytochem drug screening resistance
IT Neoplasm
(**biopsy**; method for using multicellular particulates to analyze malignant or hyperproliferative tissue)
IT Gene, animal
RL: ANT (Analyte); DGN (Diagnostic use); ANST (Analytical study); BIOL (Biological study); USES (Uses)
(cancer related, mutation in; method for using multicellular particulates to analyze malignant or hyperproliferative tissue)
IT Neuroglia
(glioblastoma; method for using multicellular particulates to analyze malignant or hyperproliferative tissue)
IT Immunoassay

(immunocytochem.; method for using multicellular particulates to analyze malignant or hyperproliferative tissue)
IT Drug resistance
Phenotypes
(marker for; method for using multicellular particulates to analyze malignant or hyperproliferative tissue)
IT **Mesothelium**
(mesothelioma; method for using multicellular particulates to analyze malignant or hyperproliferative tissue)
IT **Animal cell**
Animal tissue
Animal tissue culture
Brain, neoplasm
Cell proliferation
Chemotherapy
Computer application
Culture media
Drug resistance
Drug screening
Epithelium
Gene therapy
Genetic methods
Genotypes
Histochemistry
Human
Immunotherapy
Kidney, neoplasm
Microtiter plates
Neoplasm
Ovary, neoplasm
Process automation
Radiotherapy
Thrombosis
Tumor markers
(method for using multicellular particulates to analyze malignant or hyperproliferative tissue)
IT Estrogen receptors
Progesterone receptors
RL: ANT (Analyte); DGN (Diagnostic use); ANST (Analytical study); BIOL (Biological study); USES (Uses)
(method for using multicellular particulates to analyze malignant or hyperproliferative tissue)
IT **Lymphocyte**
(natural killer cell; method for using multicellular particulates to analyze malignant or hyperproliferative tissue)
IT Mammary gland
(neoplasm; method for using multicellular particulates to analyze malignant or hyperproliferative tissue)
IT Mutation
(of cancer related genes; method for using multicellular particulates to analyze malignant or hyperproliferative tissue)
IT Gene, animal
RL: ANT (Analyte); DGN (Diagnostic use); ANST (Analytical study); BIOL (Biological study); USES (Uses)
(oncogene; method for using multicellular particulates to analyze malignant or hyperproliferative tissue)
IT Drug tolerance
(therapeutic index; method for using multicellular particulates to analyze malignant or hyperproliferative tissue)
IT Hormones, animal, biological studies
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(therapy; method for using multicellular particulates to analyze malignant or hyperproliferative tissue)

IT Cell morphology
(tumor; method for using multicellular particulates to analyze malignant or hyperproliferative tissue)
IT 302-79-4, Retinoic acid 10540-29-1, Tamoxifen 33069-62-4, Taxol 41575-94-4, Carboplatin
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(method for using multicellular particulates to analyze malignant or hyperproliferative tissue)

L89 ANSWER 4 OF 5 HCAPLUS COPYRIGHT 2003 ACS
AN **1998:71095** HCAPLUS
DN **128:97698**
TI Precise efficacy assay methods for active agents, including chemotherapeutic agents, using cohesive multicellular particulates
IN **Kornblith, Paul L.**
PA Precision Therapeutics, Inc., USA
SO PCT Int. Appl., 16 pp.
CODEN: PIXXD2
DT Patent
LA English
IC ICM A01N001-02
ICS C12N001-02; C12N005-00; C12Q001-02; C12Q001-18; C12Q001-24
CC 1-1 (Pharmacology)
FAN.CNT 3

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9802038	A1	19980122	WO 1997-US11595	19970710
	W: AL, AM, AT, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, CZ, DE, DE, DK, DK, EE, EE, ES, FI, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
	RW: GH, KE, LS, MW, SD, SZ, UG, ZW, AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG				
	US 5728541	A	19980317	US 1996-679056	19960712
	CA 2259984	AA	19980122	CA 1997-2259984	19970710
	AU 9736493	A1	19980209	AU 1997-36493	19970710
	AU 712302	B2	19991104		
	EP 912085	A1	19990506	EP 1997-933267	19970710
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, FI				
	BR 9710348	A	20000111	BR 1997-10348	19970710
	CN 1275885	A	20001206	CN 1997-196143	19970710
	IL 127964	A1	20010724	IL 1997-127964	19970710
	JP 2002501609	T2	20020115	JP 1998-506069	19970710
	NZ 333830	A	20021220	NZ 1997-333830	19970710
	US 2002192638	A1	20021219	US 2002-205887	20020726
PRAI	US 1996-679056	A	19960712		
	WO 1997-US11595	W	19970710		
	US 1998-40161	A1	19980317		

AB An improved system is disclosed for screening a multiple of candidate therapeutic or chemotherapeutic agents for efficacy as to a specific patient, in which a tissue sample from the patient is harvested, cultured and sep. exposed to a plurality of treatments and/or therapeutic agents for the purpose of objectively identifying the best treatment or agent for the particular patient. Specific method innovations such as tissue sample prepn. techniques render this method practically as well as theor. useful. One particularly important tissue sample prepn. technique is the initial prepn. of cohesive multicellular particulates of the tissue sample, rather than enzymically dissocd. cell suspensions or prepns., for initial tissue culture monolayer prepn. By subjecting uniform samples of cells to a wide

variety of active agents (and concns. thereof), the most promising agent and concn. for treatment of a particular patient can be detd.
ST drug screening tissue culture; chemotherapeutic screening tissue culture
IT Apparatus
(Terasaki dispenser; precise efficacy assay methods for active agents, including chemotherapeutic agents, using cohesive multicellular particulates)
IT Radiotherapy
(agents for; precise efficacy assay methods for active agents, including chemotherapeutic agents, using cohesive multicellular particulates)
IT Body fluid
(effusion; precise efficacy assay methods for active agents, including chemotherapeutic agents, using cohesive multicellular particulates)
IT **Animal cell**
Animal tissue
Animal tissue culture
Antitumor agents
Ascites
Chemotherapy
Cytotoxic agents
Drug screening
Drugs
Immunotherapy
Radioprotectants
Radiosensitizers, biological
Wound healing promoters
(precise efficacy assay methods for active agents, including chemotherapeutic agents, using cohesive multicellular particulates)
IT Neoplasm
(tissue; precise efficacy assay methods for active agents, including chemotherapeutic agents, using cohesive multicellular particulates)
RE.CNT 5 THERE ARE 5 CITED REFERENCES AVAILABLE FOR THIS RECORD
RE
(1) Morgan; US 5270172 A 1993
(2) Naughton; US 5443950 A 1995 HCAPLUS
(3) Rotman; US 4937187 A 1990 HCAPLUS
(4) Stampfer; US 4423145 A 1983
(5) Yen-Maguire; US 5242806 A 1993

L89 ANSWER 5 OF 5 HCAPLUS COPYRIGHT 2003 ACS
AN 1985:3969 HCAPLUS
DN 102:3969
TI Correlation of experimental and clinical studies of metabolism by PET scanning
AU **Kornblith, Paul L.**; Cummins, C. J.; Smith, Barry H.; Brooks, Rodney A.; Patronas, Nicholas J.; Di Chiro, Giovanni
CS Dep. Health and Human Serv., Natl. Inst. Neurol. Communicative Disorders and Stroke, Bethesda, MD, USA
SO Progress in Experimental Tumor Research (1984), 27(Brain Tumor Biol.), 170-8
CODEN: PEXTAR; ISSN: 0079-6263
DT Journal
LA English
CC 14-1 (Mammalian Pathological Biochemistry)
Section cross-reference(s): **9**
AB There was a high pos. correlation between glucose uptake by human glioma measured in situ by the 18F-labeled 2-deoxyglucose positron emission tomog. (qFDG-PET) procedure and the glucose uptake measured biochem. in tissue culture lines obsd. from the same tumor. Glucose metab. was related to tumor grade, with grade-IV gliomas showing a mean rate of glucose metab. (LCMRGlu) 1.6-fold greater than that of contralateral normal brain. Grade-III gliomas had a near-normal LCMRGlu, and low-grade

gliomas had a somewhat depressed metabolic rate. Both hexokinase and phosphofructokinase (glycolysis-regulating enzymes) were elevated and glucose-6-phosphate dehydrogenase (a phosphogluconate pathway enzyme) was decreased, in high-grade gliomas. These enzyme alterations were consistent with the increased LCMRGlu obsd. in high-grade gliomas. The pathophysiol. significance of the data and applications of the FDG-PET and tissue culture methods to tumor grading, follow-up, and metab. are discussed.

ST glioma metab positron emission tomog; glucose metab glioma methodol

IT Glycolysis
(by glioma of human, positron emission tomog. vs. tissue culture in study of)

IT **Animal tissue culture**
(fro glucose metab. by glioma of human, positron emission tomog. in relation to)

IT Neoplasm, metabolism
(glucose metab. by, of brain of human, positron emission tomog. vs. tissue culture in study of)

IT Enzymes
RL: BIOL (Biological study)
(glycolytic, of glioma of human, tumor grade in relation to)

IT Radiography
(laminog., positron, computerized, for glucose metab. by glioma of human, tissue culture in relation to)

IT Neuroglia
(neoplasm, glucose metab. by human, positron emission tomog. vs. tissue culture in study of)

IT 50-99-7, biological studies
RL: BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)
(metab. of, by glioma of human, positron emission tomog. vs. tissue culture in study of)

IT 9001-40-5 9001-51-8 9001-80-3
RL: BIOL (Biological study)
(of glioma of human, glucose metab. and tumor grade in relation to)

=> d tot

L107 ANSWER 1 OF 5 HCAPLUS COPYRIGHT 2003 ACS
AN 2002:575663 HCAPLUS
DN 137:106071
TI Immortalized human keratinocyte cell line
IN Allen-Hoffmann, B. Lynn
PA USA
SO U.S. Pat. Appl. Publ., 19 pp., Cont.-in-part of U.S. Ser. No. 769,124.
CODEN: USXXCO
DT Patent
LA English
IC ICM C12Q001-70
ICS C12Q001-68; G01N033-567; C12N007-00; C12N007-01; C12N005-00; C12N005-02; C12N005-06; C12N005-08
NCL 435325000
CC **9-11 (Biochemical Methods)**
Section cross-reference(s): 1, 62, 63
FAN.CNT 4

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	US 2002102726	A1	20020801	US 2001-945136	20010831 <--
	US 6514711	B2	20030204		
	US 5989837	A	19991123	US 1998-114557	19980713 <--
	US 6214567	B1	20010410	US 1999-277295	19990326 <--

US 2001023061 A1 20010920 US 2001-769124 20010124
US 6485724 B2 20021126
PRAI US 1998-114557 A1 19980713 <--
US 1999-277295 A1 19990326
US 2001-769124 A2 20010124

AB The invention concerns a method for testing the effects of various factors on human skin equiv. is disclosed. The method involves providing a human stratified squamous epithelial cell culture of an immortalized human keratinocyte cell line that forms a reconstructed epidermis, exposing the reconstructed epidermis to a factor and evaluating the effect of the factor on the reconstructed epidermis. A method for selecting preventive or therapeutic agents for skin damages caused by a factor using the same human stratified squamous epithelial cell culture system is also disclosed.

ST immortalized keratinocyte skin culture drug screening green fluorescent protein

IT **Animal cell line**
(ATCC CRL-12191; immortalized human keratinocyte cell line)

IT **Animal cell line**
(BC-1-Ep/SL; immortalized human keratinocyte cell line)

IT Pressure
(air; immortalized human keratinocyte cell line)

IT Cosmetics
(creams; immortalized human keratinocyte cell line)

IT **Animal cell**
(damage to; immortalized human keratinocyte cell line)

IT Skin
(epidermis; immortalized human keratinocyte cell line)

IT Gene, animal
RL: BSU (Biological study, unclassified); PRP (Properties); BIOL (Biological study)
(exogenous; immortalized human keratinocyte cell line)

IT Emulsions
Liquids
(form; immortalized human keratinocyte cell line)

IT Proteins
RL: BSU (Biological study, unclassified); PRP (Properties); BIOL (Biological study)
(green fluorescent; immortalized human keratinocyte cell line)

IT Transformation, neoplastic
(immortalization; immortalized human keratinocyte cell line)

IT **Animal tissue culture**
Cell death
Cell differentiation
Cell proliferation
Chromosome
Climate
Combinatorial library
Cosmetics
DNA sequence analysis
Detergents
Drug screening
Drugs
Fibroblast
Genetic methods
Histochemistry
Human
Microscopy
Skin
Surfactants
Toxicity
UV radiation
(immortalized human keratinocyte cell line)

IT Reporter gene
RL: BSU (Biological study, unclassified); PRP (Properties); BIOL (Biological study)
(immortalized human keratinocyte cell line)
IT Collagens, uses
RL: NUU (Other use, unclassified); USES (Uses)
(immortalized human keratinocyte cell line)
IT Bases, biological studies
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(immortalized human keratinocyte cell line)
IT Chemicals
(industrial; immortalized human keratinocyte cell line)
IT Acids, biological studies
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(inorg.; immortalized human keratinocyte cell line)
IT Skin
(keratinocyte; immortalized human keratinocyte cell line)
IT Bases, biological studies
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(org.; immortalized human keratinocyte cell line)
IT **Epithelium**
(stratified squamous; immortalized human keratinocyte cell line)
IT **Animal cell**
(survival of; immortalized human keratinocyte cell line)
IT 1746-01-6, TCDD
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(immortalized human keratinocyte cell line)

L107 ANSWER 2 OF 5 HCAPLUS COPYRIGHT 2003 ACS
AN 2001:703739 HCAPLUS
DN 135:269653
TI Use of multipotent neural stem cells and their progeny for the screening of drugs and other biological agents
IN Weiss, Samuel; Reynolds, Brent; Hammang, Joseph P.; Baetge, E. Edward
PA Neurospheres Holdings, Ltd., Can.
SO U.S., 42 pp., Cont.-in-part of U.S. Ser. No. 385,404, abandoned.
CODEN: USXXAM
DT Patent
LA English
IC G01N033-554; C12N005-00
NCL 435007210
CC **9-11** (**Biochemical Methods**)
Section cross-reference(s): 1, 13, 14
FAN.CNT 8

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PI	US 6294346	B1	20010925	US 1995-484406	19950607	<--
	WO 9301275	A1	19930121	WO 1992-CA283	19920707	<--
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	RW: AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG					

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             SE, SK, UA, VN
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     EP 728194          B1   20030108
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     NO 9601859         A    19960703      NO 1996-1859     19960508 <--
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PRAI US 1991-726812     B1   19910708  <--
     US 1992-961813     B1   19921016  <--
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US 1994-359345	A	19941220	<--
US 1995-481893	A	19950607	<--
EP 1995-931864	A3	19950922	<--

AB A culture method for detg. the effect of a biol. agent on multipotent neural stem cell progeny is provided. In the presence of growth factors, multipotent neural stem cells are induced to proliferate in culture. The multipotent neural stem cells may be obtained from normal neural tissue or from a donor afflicted with a disease such as Alzheimer's Disease, Parkinson's Disease or Down's Syndrome. At various stages in the differentiation process of the multipotent neural stem cell progeny, the effects of a biol. agent, such as a virus, protein, peptide, amino acid, lipid, carbohydrate, nucleic acid or a drug or pro-drug on cell activity are detd. Addnl., a method of screening the effects of biol. agents on a clonal population of neural cells is provided. The technol. provides an efficient method for the generation of large nos. of pre- and post-natal neural cells under controlled, defined conditions. The disclosed cultures provide an optimal source of normal and diseased neural cells at various developmental stages, which can be screened for potential side effects in addn. to testing the action and efficacy of different biol. agents.

ST stem cell neuron culture drug screening differentiation proliferation factor

IT Gene, animal
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(NGF; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)

IT Disease models
(Parkinson's; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)

IT Neurotrophic factor receptors
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(TrkB; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)

IT Gene, microbial
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(b-galactosidase; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)

IT Gene, animal
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(bcl-2; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)

IT Neurotrophic factors
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(brain-derived; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)

IT Transcription factors
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(c-fos, pathway inhibitors; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)

IT Embryo, animal
(fetus; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)

IT Neurotrophic factors
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(glial-derived; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT Immunoassay
(immunocytochem.; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT Chemokines
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(macrophage inflammatory protein; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT GAP-43 (protein)
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(membrane phosphoprotein; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT Transplant and Transplantation
(neural stem cell; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT Nerve
(neuroblast, neurosphere; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT Nerve
(neuron; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT Cell
(stem, neural; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT Cytotoxic agents
(tyrphostins, interfere with c-fos path activation; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT Alzheimer's disease
Animal tissue culture
Astrocyte
Cell differentiation
Cell proliferation
Down's syndrome
Drug screening
Genetic methods
Mammal (Mammalia)
Neuroglia
Parkinson's disease
Virus
(use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT Amino acids, biological studies
Carbohydrates, biological studies
Ciliary neurotrophic factor
Hormones, animal, biological studies
Lipids, biological studies
Macrophage inflammatory protein 1.alpha.
Macrophage inflammatory protein 1.beta.
Macrophage inflammatory protein 2
Nucleic acids
Peptides, biological studies
Platelet-derived growth factors
Proteins, general, biological studies
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)

IT **Myelin**
RL: BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)
(use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT **Transforming growth factors**
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(.**alpha**.-; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT **Transforming growth factors**
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(.**beta**.-; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT 62031-54-3, FGF
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(b; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT 80449-02-1, Tyrosine kinase
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(inhibitors; use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
IT 179047-86-0 179047-88-2 179047-90-6 179047-91-7 222586-28-9, 1: PN: US6294346 SEQID: 1 unclaimed DNA 222586-30-3, 3: PN: US6294346 SEQID: 3 unclaimed DNA 222586-32-5, 5: PN: US6294346 SEQID: 5 unclaimed DNA 222586-34-7, 7: PN: US6294346 SEQID: 7 unclaimed DNA
RL: PRP (Properties)
(unclaimed nucleotide sequence; use of multipotent neural stem cells and their progeny for the screening of drugs and other biol. agents)
IT 9050-30-0, Heparan sulfate 9061-61-4, NGF 16561-29-8, Phorbol 12-myristate 13-acetate 24305-27-9, TRH 62229-50-9, EGF 62229-50-9D, EGF, liganded derivs. 62996-74-1, Staurosporine 67763-96-6, IGF-1 106096-92-8, FGF-1 106096-93-9, FGF-2 114949-22-3, Activin 120685-11-2, CGP-41251
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); BIOL (Biological study)
(use of multipotent neural stem cells and progeny for screening of drugs and other biol. agents)
RE.CNT 121 THERE ARE 121 CITED REFERENCES AVAILABLE FOR THIS RECORD
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L107 ANSWER 3 OF 5 HCAPLUS COPYRIGHT 2003 ACS
AN 2001:464263 HCAPLUS
DN 135:58181
TI Metastasis models using green fluorescent protein (gfp) as a marker
IN Tan, Yuying; Chishima, Takashi
PA AntiCancer, Inc., USA
SO U.S., 11 pp., Cont.-in-part of U.S. Ser. No. 67,734.
CODEN: USXXAM
DT Patent
LA English
IC ICM A61K035-00
ICS A61K048-00; C12N015-63; C12N015-85
NCL 424093210
CC **9-16 (Biochemical Methods)**
Section cross-reference(s): 1, 14

FAN.CNT 5

	PATENT NO.	KIND	DATE		APPLICATION NO.	DATE	
PI	US 6251384	B1	20010626		US 1999-226856	19990107	<--
	US 6232523	B1	20010515		US 1997-848539	19970428	<--
	US 6235967	B1	20010522		US 1998-49544	19980327	<--
	US 6235968	B1	20010522		US 1998-67734	19980428	<--
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	WO 2000040274	A1	20000713		WO 2000-US243	20000107	
	W: AU, CA, JP						
	RW: AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE						
	EP 1156833	A1	20011128		EP 2000-902334	20000107	
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, FI						
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PRAI	US 1997-848539	A2	19970428	<--			
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	WO 2000-US243	W	20000107				

AB A method to follow the progression of metastasis of a primary tumor, which method comprises removing fresh organ tissues from a vertebrate subject which has been modified to contain tumor cells that express GFP and observing the excised tissues for the presence of fluorescence is disclosed. The fluorescence can also be monitored by observing the tissues in situ. Vertebrate subjects which contain GFP producing tumors are useful models to study the mechanism of metastasis, as well as to evaluate candidate protocols and drugs. In addn., subjects already harboring tumors can be treated so as to modify the endogenous tumors to contain GFP. This permits clin. applications. Finally, by injecting a contrast dye into a subject harboring a GFP-labeled tumor, angiogenesis in the tumor can be obsd. directly.

ST metastasis model green fluorescence protein marker

IT **Animal cell line**
(CHO-K1; metastasis models using green fluorescent protein as a marker)

IT Dyes
(Contrast; metastasis models using green fluorescent protein as a marker)

IT Gene
(expression; metastasis models using green fluorescent protein as a marker)

IT Imaging
(fluorescent, optical tumor; metastasis models using green fluorescent protein as a marker)

IT Proteins, specific or class
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(green fluorescent; metastasis models using green fluorescent protein as a marker)

IT Angiogenesis
Animal cell
Animal cell line
Animal tissue
Disease models
Drug screening
Fluorescence
Lung, neoplasm
Mammal (Mammalia)
Microscopy
Mouse
Organ, animal

Retroviral vectors
Tumor markers
Vertebrate (Vertebrata)
Virus vectors
(metastasis models using green fluorescent protein as a marker)
IT Neoplasm
(metastasis; metastasis models using green fluorescent protein as a marker)
IT Surgery
(orthotopic implantation; metastasis models using green fluorescent protein as a marker)
RE.CNT 65 THERE ARE 65 CITED REFERENCES AVAILABLE FOR THIS RECORD
RE
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L107 ANSWER 4 OF 5 HCAPLUS COPYRIGHT 2003 ACS
AN 1999:748234 HCAPLUS
DN 131:319888
TI Immortalized human keratinocyte cell line
IN Allen-Hoffmann, B. Lynn; Schlosser, Sandra J.; Pickart, Michael A.
PA Wisconsin Alumni Research Foundation, USA
SO U.S., 19 pp.
CODEN: USXXAM
DT Patent
LA English
IC ICM G01N033-567
ICS C12N005-08
NCL 435007210
CC **9-11** (**Biochemical Methods**)
Section cross-reference(s): 1, 14
FAN.CNT 4

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE	
PI	US 5989837	A	19991123	US 1998-114557	19980713	<--
	US 6214567	B1	20010410	US 1999-277295	19990326	<--
	CA 2336717	AA	20000120	CA 1999-2336717	19990712	<--
	WO 2000003244	A2	20000120	WO 1999-US15671	19990712	<--
	WO 2000003244	A3	20010426			
	W: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM					
	RW: GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG					
	AU 9949850	A1	20000201	AU 1999-49850	19990712	<--
	BR 9912047	A	20010403	BR 1999-12047	19990712	<--
	EP 1117423	A2	20010725	EP 1999-933896	19990712	<--
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	JP 2002520594	T2	20020709	JP 2000-559428	19990712	<--
	NZ 509184	A	20030131	NZ 1999-509184	19990712	<--
	US 2002102726	A1	20020801	US 2001-945136	20010831	<--
	US 6514711	B2	20030204			
	US 2002192196	A1	20021219	US 2002-131977	20020424	<--
PRAI	US 1998-114557	A1	19980713	<--		

US 1999-277295 A1 19990326
WO 1999-US15671 W 19990712
US 2001-769124 A2 20010124
US 2001-286169P P 20010424
US 2001-844194 A2 20010427

AB A spontaneously immortalized human keratinocyte cell line is disclosed. In a preferred embodiment, this cell line is ATCC 12191. In another embodiment of the invention, a method of assaying the effect of a test tumor cell modulation agent is disclosed. The method comprises the steps of obtaining a human stratified squamous epithelial cell culture, wherein the culture comprises human malignant squamous epithelial cells and spontaneously immortalized human keratinocytes, wherein the culture forms a reconstituted epidermis. One then treats the epidermis with a test tumor cell modulation agent and evaluates the growth of the malignant cells within the epidermis.

ST immortalized keratinocyte cell line

IT **Animal cell line**
(EC; immortalized human keratinocyte cell line)

IT Gene
RL: BPR (Biological process); BSU (Biological study, unclassified); BUU (Biological use, unclassified); BIOL (Biological study); PROC (Process); USES (Uses)
(Heterologous; immortalized human keratinocyte cell line)

IT Films
(bio-; immortalized human keratinocyte cell line)

IT Skin
(epidermis; immortalized human keratinocyte cell line)

IT Proteins, specific or class
RL: BPR (Biological process); BSU (Biological study, unclassified); BIOL (Biological study); PROC (Process)
(green fluorescent; immortalized human keratinocyte cell line)

IT Transformation, neoplastic
(immortalization; immortalized human keratinocyte cell line)

IT **Animal cell line**
Animal tissue culture
Drug screening
Growth, animal
Toxicity
Transformation, genetic
(immortalized human keratinocyte cell line)

IT **Genetic markers**
RL: BPR (Biological process); BSU (Biological study, unclassified); BUU (Biological use, unclassified); BIOL (Biological study); PROC (Process); USES (Uses)
(immortalized human keratinocyte cell line)

IT Skin
(keratinocyte; immortalized human keratinocyte cell line)

IT **Animal tissue culture**
(monolayer; immortalized human keratinocyte cell line)

IT **Animal tissue culture**
(organotypic; immortalized human keratinocyte cell line)

IT **Epithelium**
(stratified squamous; immortalized human keratinocyte cell line)

RE.CNT 9 THERE ARE 9 CITED REFERENCES AVAILABLE FOR THIS RECORD

RE

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L107 ANSWER 5 OF 5 HCAPLUS COPYRIGHT 2003 ACS
AN 1999:450863 HCAPLUS
DN 131:99526
TI Immortalized human bone marrow endothelial cell line and their adhesion to cancer cells and uses in treatment of metastasis
IN Pienta, Kenneth J.
PA The Regents of the University of Michigan, USA
SO U.S., 13 pp.
CODEN: USXXAM
DT Patent
LA English
IC ICM G01N033-53
ICS C12N005-00
NCL 435007230
CC **9-11 (Biochemical Methods)**
Section cross-reference(s): 13, 63
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	US 5925531	A	19990720	US 1997-956844	19971023 <--
PRAI	US 1997-956844		19971023 <--		

AB The present invention provides immortalized human bone marrow endothelial cells which are useful for the study of tumor metastasis. Primary bone marrow endothelial cells from a 25-yr-old Caucasian man were immortalized with SV40 large T antigen to create the HBME-1 cell line. Karyotyping revealed a heterogeneous karyotype with both diploid and hyper-tetraploid populations of cells. The cells adhere to cancer cells, are easily harvested from tissue culture by trypsinization, and grow well in std. DMEM supplemented with 10% FBS. In particular, the human bone marrow endothelial cell lines provided by the invention provide an in vitro model system for screening compds. for the ability to reduce, prevent, or inhibit the metastasis of cancer cells to bone tissue.
ST bone marrow epithelium cell immortalization; cancer cell adhesion bone marrow epithelium cell; metastasis bone marrow epithelium cell
IT **Animal cell line**
(HBME-1; immortalized human bone marrow endothelial cell line and their adhesion to cancer cells and uses in treatment of metastasis)
IT Intestine, neoplasm
(colon; immortalized human bone marrow endothelial cell line and their adhesion to cancer cells and uses in treatment of metastasis)
IT Agglutinins and Lectins
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(galactose-binding, galectin, screening compds. for modulating binding of epithelial and cancer cells; immortalized human bone marrow endothelial cell line and their adhesion to cancer cells and uses in treatment of metastasis)
IT Transformation, neoplastic
(immortalization; immortalized human bone marrow endothelial cell line and their adhesion to cancer cells and uses in treatment of metastasis)
IT **Bone marrow**
Cell adhesion
Disease models
Drug screening
Neoplasm
(immortalized human bone marrow endothelial cell line and their adhesion to cancer cells and uses in treatment of metastasis)
IT Antigens
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES

(Uses)
(large T, treatment with SV40 large T antigen for cell line prepn.; immortalized human bone marrow endothelial cell line and their adhesion to cancer cells and uses in treatment of metastasis)

IT **Antitumor agents**
Neoplasm
(metastasis; immortalized human bone marrow endothelial cell line and their adhesion to cancer cells and uses in treatment of metastasis)

IT Mammary gland
Prostate gland
(neoplasm; immortalized human bone marrow endothelial cell line and their adhesion to cancer cells and uses in treatment of metastasis)

IT RGD peptides
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(screening compds. for modulating binding of epithelial and cancer cells; immortalized human bone marrow endothelial cell line and their adhesion to cancer cells and uses in treatment of metastasis)

IT 99896-85-2
RL: BAC (Biological activity or effector, except adverse); BSU (Biological study, unclassified); THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(screening compds. for modulating binding of epithelial and cancer cells; immortalized human bone marrow endothelial cell line and their adhesion to cancer cells and uses in treatment of metastasis)

RE.CNT 33 THERE ARE 33 CITED REFERENCES AVAILABLE FOR THIS RECORD

RE

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=> d his

     (FILE 'HOME' ENTERED AT 08:40:50 ON 02 APR 2003)
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     FILE 'HCAPLUS' ENTERED AT 08:41:09 ON 02 APR 2003
L1          6600 S PLASMINOGEN(L)ACTIVAT?(L)INHIBIT?(L)1
L2          3865 S PAI 1
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L4           959 S PLASMINOGEN ACTIVAT? INHIBIT? TYPE 1
L5             1 S EUROKINASE (S) PLASMINOGEN (S) ACTIVAT?
L6          5790 S UROKINASE (S) PLASMINOGEN (S) ACTIVAT?
L7          1709 S UROKINASE PLASMINOGEN ACTIVAT?
L8          3520 S UROKINASE TYPE PLASMINOGEN ACTIVAT?
L9          5919 S ALPHA(L)FETOPROTEIN
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                 E E4+ALL
L10         4980 S E3,E2+NT
L11         4255 S CARCINOEMBRYO? (L) ANTIGEN?
                 E CARCINOEMBRYONIC ANTIGEN/CT
                 E E3+ALL
L12         3370 S E9,E10,E8+NT
L13         9449 S TRANSFORMING(L)GROWTH(L)FACTOR(L)ALPHA
L14         3747 S TRANSFORMING GROWTH FACTOR ALPHA
                 E TRANSFORMING GROWTH FACTOR/CT
                 E E4+ALL
                 E E2+ALL
L15         4433 S E5,E6,E4
L16         6164 S TGF(S)ALPHA
L17          184 S TRANSFORMING(L)GROWTH(L)SUBSTANCE(L)ALPHA
L18        21973 S TRANSFORMING(L)GROWTH(L)FACTOR(L)BETA
L19        19473 S TRANSFORMING GROWTH FACTOR BETA
                 E TRANSFORMING GROWTH FACTOR BETA/CT
                 E TRANSFORMING GROWTH FACTOR .BETA./CT
                 E E3+ALL
                 E E2+ALL
L20        14578 S E5,E6,E4+NT
L21        21730 S BETA(S)TRANSFORMING(S)GROWTH(S)FACTOR
L22          232 S BETA(S)TRANSFORMING(S)GROWTH(S)SUBSTANCE
                 E ANIMAL GROWTH SUBSTANCES/CT
                 E E3+ALL
                 E ANIMAL GROWTH SUBSTANCES/CT
                 E E4+ALL
L23         2183 S E1,E2
L24        10896 S E3,E4
L25        20842 S MAJOR(S)HISTOCOMPATIB?(S)COMPLEX
L26        18914 S MAJOR HISTOCOMPATIB? COMPLEX
                 E MAJOR HISTOCOMPATIBILITY COMPLEX/CT
                 E MAJOR HISTOCOMPATIBILITY/CT
                 E HITOCOMPATIBILITY/CT
                 E HISTOCOMPATIBILITY/CT
L27        10894 S E99-E106
L28        17309 S E6-E98,E107-E117
                 E E5+ALL
L29        35914 S E4,E3+NT

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L30            2 S 140208-23-7 OR 9039-53-6
L31           93 S PLASMINOGEN(S)ACTIVATOR(S)INHIBITOR
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L33          111 S ALPHA(S)FETOPROTEIN
L34          385 S CARCINOEMBRYONIC(S)ANTIGEN
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L35         303 S TRANSFORMING(S)GROWTH(S)FACTOR(S)ALPHA
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L37        1268 S MAJOR(S)HISTOCOMPATIBILITY(S)COMPLEX

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                E E5+ALL
L40        3998 S E2
L41        6727 S TUMOR ANTIGEN OR TUMOR ASSOC? ANTIGEN
                E CELL MARKER/CT
                E CELLULAR MARKER/CT
L42        2267 S (CELL OR CELLULAR)()MARKER
L43       23225 S SECRET?(S)FACTOR
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L45         252 S SECRET?(S)GROWTH(S)SUBSTANCE
L46      121483 S L39-L45
                E BIOPSY/CT
                E BIOPSY
                E BIOP
                E BIOPS
L47       24524 S E11-E23
L48           8 S E29-E32
L49        1065 S L46 AND L47,L48
L50        4256 S L46 AND ?MALIGN?
                E CULTURE/CT
L51        8429 S E4-E10
                E E10+ALL
                E E2+ALL
L52        8287 S E2,E1+NT
                E E10+ALL
L53       29944 S E2,E3,E1+NT
                E E9+ALL
L54      903575 S E3,E2+NT
L55       40371 S L46 AND L51-L54
L56        1551 S L49,L50 AND L55
                E DRUG SCREENING/CT
L57         886 S E4,E5
                E E3+ALL
L58       23428 S E2,E1
L59          85 S E7
                E E5+ALL
L60        5879 S E3+NT
L61        3660 S E12+NT OR E14+NT
L62        3193 S E16+NT
L63        1030 S L46 AND L57-L62
L64         502 S L63 AND L55
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                E E1+ALL
L66       76958 S E1
                E E2+ALL
L67       79405 S E3+NT
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L69          16 S L68 AND L63
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L70          47 S E3-E7
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L77            5 S L75 NOT CEREBELLAR/TI
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                 SEL DN AN 3
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L81            5 S L80 AND L38-L69
                 E TUMOR MARKER/CT
                 E E4+ALL
L82        11274 S E2+NT
L83        30681 S E1+NT
L84            1 S L80 AND L82,L83
L85            5 S L81,L84
                 E ANIMAL TISSUE/CT
L86       327587 S E3+NT
                 E E3+ALL
L87            2 S E3
L88            4 S L70 AND L86,L87
L89            5 S L85,L88

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L92         6937 S L90 AND L91
L93         1284 S L92 AND (BIOCHEM?(L)METHOD?)/SC,SX
L94          286 S L93 AND L66,L67
L95          157 S L93 AND L46
L96          141 S L93 AND L82,L83
L97           97 S L94-L96 AND (PY<=1998 OR PRY<=1998 OR AY<=1998)
L98           52 S L97 NOT 3/SC,SX
L99           50 S L98 NOT L89
L100          23 S L99 AND 9/SC
                 E EPITHELIUM/CT
                 E E3+ALL
L101       14857 S E3+NT
L102          65 S L101 AND L93
L103          16 S L102 AND (PY<=1998 OR PRY<=1998 OR AY<=1998)
L104          14 S L103 NOT L89
L105          36 S L100,L104
L106          23 S L99 NOT L105
L107           5 S (137:106071 or 135:269653 or 135:58181 or 131:319888 or
=> fil medline
FILE 'MEDLINE' ENTERED AT 10:41:56 ON 08 APR 2003

 FILE LAST UPDATED: 6 APR 2003 (20030406/UP).  FILE COVERS 1958 TO DATE.

 On June 9, 2002, MEDLINE was reloaded.  See HELP RLOAD for details.

 MEDLINE thesauri in the /CN, /CT, and /MN fields incorporate the
 MeSH 2003 vocabulary.  See http://www.nlm.nih.gov/mesh/summ2003.html
 for a description on changes.

 This file contains CAS Registry Numbers for easy and accurate
 substance identification.

=> d all tot

L84  ANSWER 1 OF 8     MEDLINE
AN   2003098807     MEDLINE
DN   22498380   PubMed ID: 12610871
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TI Histoculture drug response assay (HDRA) guided induction concurrent chemoradiotherapy for mediastinal node-positive non-small cell lung cancer.
AU Yoshimasu Tatsuya; Oura Shoji; Hirai Issei; Kokawa Yozo; Sasaki Rie; Honda Kentaro; Tanino Hirokazu; Sakurai Teruhisa; Okamura Yoshitaka
CS Dept. of Thoracic and Cardiovascular Surgery, Wakayama Medical University.
SO GAN TO KAGAKU RYOHO [JAPANESE JOURNAL OF CANCER AND CHEMOTHERAPY], (2003 Feb) 30 (2) 231-5.
Journal code: 7810034. ISSN: 0385-0684.
CY Japan
DT Journal; Article; (JOURNAL ARTICLE)
LA Japanese
FS Priority Journals
EM 200303
ED Entered STN: 20030304
Last Updated on STN: 20030311
Entered Medline: 20030310
AB To improve the response to chemotherapy for non-small cell lung cancer (NSCLC), effective drugs should be selected for each patient. In 1994 we introduced histoculture drug response assay (HDRA) for NSCLC patients. For clinical N2 patients, **biopsy** of mediastinal lymph node is performed both for histological diagnosis and for HDRA. Induction concurrent chemoradiotherapy is then performed using HDRA positive chemotherapy agents. We have treated three patients with this strategy. HDRA could be performed using mediastinal lymph node **biopsy** specimens. Tumor reduction rates of these patients were 80.4%, 85.3%, and 57.1%. Their histological responses were Ef.3, Ef.2, and Ef.1b, respectively. Complete resection was done in all patients. This strategy appeared to be useful in NSCLC patients with mediastinal lymph node metastasis.
CT Check Tags: Female; Human; Male
Aged
Antineoplastic Combined Chemotherapy Protocols: AD, administration & dosage
***Antineoplastic Combined Chemotherapy Protocols: TU, therapeutic use**
***Carcinoma, Non-Small-Cell Lung: DT, drug therapy**
Carcinoma, Non-Small-Cell Lung: RT, radiotherapy
Carcinoma, Non-Small-Cell Lung: SU, surgery
Cisplatin: AD, administration & dosage
Combined Modality Therapy
Drug Screening Assays, Antitumor
English Abstract
Etoposide: AD, administration & dosage
***Lung Neoplasms: DT, drug therapy**
***Lung Neoplasms: RT, radiotherapy**
Lung Neoplasms: SU, surgery
Lymphatic Metastasis
Middle Age
Paclitaxel: AD, administration & dosage
*Paclitaxel: AA, analogs & derivatives
Pneumonectomy
Tumor Cells, Cultured
RN 114977-28-5 (docetaxel); 15663-27-1 (Cisplatin); 33069-62-4 (Paclitaxel); 33419-42-0 (Etoposide)
CN 0 (Antineoplastic Combined Chemotherapy Protocols); 0 (VP-P protocol)

L84 ANSWER 2 OF 8 MEDLINE
AN **92253979** MEDLINE
DN **92253979** PubMed ID: **1579821**
TI [Resistance of cancer cells to antineoplastic agents. Can the efficiency of chemotherapy be predicted?].
Resistance des cellules cancereuses aux agents antineoplasiques. Peut-on

predire l'efficacite d'une chimiotherapie?.
AU Benard J
SO REVUE DU PRATICIEN, **(1992 Feb 1)** 42 (3) 318-9. Ref: 8
Journal code: 0404334. ISSN: 0035-2640.
CY France
DT Editorial
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LA French
FS Foreign
EM 199206
ED Entered STN: 19920619
Last Updated on STN: 19970203
Entered Medline: 19920611
CT Check Tags: Human
***Antineoplastic Agents: PD, pharmacology**
Drug Resistance
Neoplasms: GE, genetics
***Tumor Cells, Cultured: DE, drug effects**
Tumor Markers, Biological: GE, genetics
CN 0 (Antineoplastic Agents); 0 (Tumor Markers, Biological)

L84 ANSWER 3 OF 8 MEDLINE
AN **91274143** MEDLINE
DN **91274143** PubMed ID: **2054260**
TI Three-dimensional histoculture: origins and applications in cancer research.
AU Hoffman R M
CS Laboratory of Cancer Biology, University of California, San Diego, La Jolla 92093-0609.
SO CANCER CELLS, **(1991 Mar)** 3 (3) 86-92. Ref: 75
Journal code: 9000382. ISSN: 1042-2196.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
(REVIEW, TUTORIAL)
LA English
FS Priority Journals
EM 199108
ED Entered STN: 19910818
Last Updated on STN: 19970203
Entered Medline: 19910801
AB The ability to grow cells in monolayer culture has afforded investigators the opportunity to study many aspects of cancer cell biology under carefully controlled conditions. Nonetheless, an important factor that has often been overlooked is that cells in this configuration undergo a loss of structural integrity that may significantly alter their functional properties. Three-dimensional histoculture represents a useful alternative approach to monolayer culture because it preserves the native architecture of cells while still allowing ease of experimental manipulation. This review discusses the origins of three-dimensional cultures, the potential application of these cultures to assays of tumor cell metastasis and drug sensitivity, and the evidence from gene expression studies that these cultures may be more realistic tumor models than cell monolayers.
CT Check Tags: Animal; Human
Antigens, Neoplasm: BI, biosynthesis
Cell Aggregation
Cell Movement
Cellulose
Collagen
Drug Screening Assays, Antitumor
Gene Expression Regulation, Neoplastic
Neoplasm Invasiveness: PA, pathology

Neoplasm Metastasis: PA, pathology
Organ Culture: IS, instrumentation
Organ Culture: MT, methods
Tissue Culture: IS, instrumentation
***Tissue Culture: MT, methods**
Tumor Cells, Cultured: DE, drug effects
Tumor Cells, Cultured: IM, immunology
***Tumor Cells, Cultured: PA, pathology**
RN 9004-34-6 (Cellulose); 9007-34-5 (Collagen)
CN 0 (Antigens, Neoplasm)

L84 ANSWER 4 OF 8 MEDLINE
AN **90141259** MEDLINE
DN **90141259** PubMed ID: **401249**
TI "Invasiveness" in tissue culture: a technique for study of gliomas.
AU Scott R M; Liszczak T M; **Kornblith P L**
CS Department of Neurosurgery, Tufts University School of Medicine, Boston.
SO SURGICAL FORUM, (1978) 29 531-3.
Journal code: 0337723. ISSN: 0071-8041.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 199002
ED Entered STN: 19900328
Last Updated on STN: 19900328
Entered Medline: 19900226
CT Check Tags: Human
***Glioma: PA, pathology**
Methods
Neoplasm Invasiveness
Tissue Culture

L84 ANSWER 5 OF 8 MEDLINE
AN **88080034** MEDLINE
DN **88080034** PubMed ID: **3121170**
TI Plasminogen activator and inhibitor activity in human glioma cells and modulation by sodium butyrate.
AU Gross J L; Behrens D L; Mullins D E; **Kornblith P L**; Dexter D L
CS Medical Products Department, E.I. Du Pont de Nemours & Co., Inc., Wilmington, Delaware 19898.
NC CA23753 (NCI)
SO CANCER RESEARCH, (1988 Jan 15) 48 (2) 291-6.
Journal code: 2984705R. ISSN: 0008-5472.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 198802
ED Entered STN: 19900305
Last Updated on STN: 19990129
Entered Medline: 19880223
AB The activity of the serine protease plasminogen activator (PA), which correlates with tumorigenicity and metastatic capacity, was examined using the 125I-labeled fibrin plate assay in cell extracts from four human glioma lines as a function of growth in vitro. Cell-associated inhibitory activity to plasmin and urokinase-type PA was also measured concurrently. The relative PA activities differed markedly among the lines, whereas inhibitory activities did not. Two lines, SNB-19 and SNB-75, exhibited maximal PA activities (1-6 m Plough units/micrograms protein) as cultures approached confluence, whereas two other lines, SNB-56 and SNB-78, expressed low PA activity at all times (less than 0.2 m Plough units/micrograms protein). The PA of SNB-19 cell extracts was

predominantly urokinase-type PA. In addition to having the highest PA levels, SNB-19 and SNB-75 were the most clonogenic in soft agar and tumorigenic in nude mice. In contrast, SNB-56 and SNB-78 were poorly clonogenic in soft agar and were not tumorigenic in nude mice. Measured directly, inhibitory activities to plasmin, urokinase-type PA, and tissue-type PA were detected in SNB-19 (high PA) and SNB-56 (low PA) cell extracts. However, there were no qualitative or quantitative differences in inhibitor effects between SNB-19 and SNB-56 suggesting that the differences in PA activity between these lines resulted from changes in PA activity and were not due to differential plasminogen activator inhibitor effects. The ability of the differentiating agent sodium butyrate (NaB) to modulate total PA activity was also examined. Peak SNB-19 cell PA activity was decreased in a concentration (Ki, 0.75 mM) and time-dependent manner by the addition of nontoxic amounts of NaB. The dose-dependent decrease in PA activity induced by NaB was most likely due to an effect on PA itself, since the action of inhibitor on urokinase was unchanged in response to NaB. These results suggest that net cellular PA activity in glioma cells is a balance between relative PA activity and inhibitor(s) effects and that this balance can be modulated by sodium butyrate.

CT Check Tags: Animal; Human; Support, U.S. Gov't, P.H.S.
*Butyrates: PD, pharmacology
Butyric Acid
*Butyric Acids: PD, pharmacology
Dose-Response Relationship, Drug
***Glioma: AN, analysis**
Glioma: GE, genetics
Glioma: PA, pathology
*Glycoproteins: AN, analysis
Mice
Mice, Nude
Neoplasm Transplantation
*Plasminogen Activators: AN, analysis
Plasminogen Activators: AI, antagonists & inhibitors
Plasminogen Inactivators
Transplantation, Heterologous
Tumor Cells, Cultured

RN 107-92-6 (Butyric Acid)

CN 0 (Butyrates); 0 (Butyric Acids); 0 (Glycoproteins); 0 (Plasminogen Inactivators); EC 3.4.21.- (Plasminogen Activators)

L84 ANSWER 6 OF 8 MEDLINE

AN **85296751** MEDLINE

DN **85296751** PubMed ID: **4033960**

TI Role of cell and explant culture in the diagnosis and characterization of human pituitary tumours.

AU Adams E F; Mashiter K

SO NEUROSURGICAL REVIEW, **(1985)** 8 (3-4) 135-40.
Journal code: 7908181. ISSN: 0344-5607.

CY GERMANY, WEST: Germany, Federal Republic of

DT Journal; Article; (JOURNAL ARTICLE)

LA English

FS Priority Journals

EM 198510

ED Entered STN: 19900320
Last Updated on STN: 19900320
Entered Medline: 19851022

AB The pattern of hormone **secretion** by human pituitary tumours in cell or explant culture has been shown to be of value in establishing the nature of the tissue. There was complete agreement between the diagnosis reached by conventional immunocytochemical techniques and by examining the **secretion** of hormones in culture. Culture techniques, however, have some advantages over immunocytochemical analysis. In particular, immunocytochemical techniques can only be used to examine a small,

possibly unrepresentative, portion of the pituitary tissue, whereas the in vitro culture systems make use of the whole tissue. In addition, in vitro culture is simpler to employ and can be carried out relatively rapidly. Cell and explant culture was therefore used to examine the nature of human pituitary tumours. To determine the incidence of mixed GH-PRL **secreting** pituitary tumours in acromegaly, the pattern of hormone **secretion** in vitro by 98 somatotrophic tumours was examined. Thirty-seven per cent were found to be pure somatotrophic tumours and 59.2% **secreted** both GH and PRL, but no other hormone, indicating that these tumours were of mixed nature. This latter group could be divided into those removed from patients with hyperprolactinaemia (35.7% of all tumours) and those from patients with normal pre-operative serum PRL levels (23.5%). A further small group (3.1%) of tumours **secreted** only GH in culture, despite elevated pre-operative serum PRL levels, indicating that the hyperprolactinaemia in these patients was due to pituitary stalk compression by the somatotrophic tumour, thereby preventing prolactin release inhibiting **factor** reaching the lactotrophs and allowing uncontrolled PRL **secretion**.(ABSTRACT TRUNCATED AT 250 WORDS)

CT Check Tags: Human; In Vitro
Gonadotropins: SE, secretion
***Pituitary Neoplasms: DI, diagnosis**
Pituitary Neoplasms: SE, secretion
Prolactin: BL, blood
Prolactin: SE, secretion
Somatotropin: SE, secretion
***Tissue Culture**

RN 9002-62-4 (Prolactin); 9002-72-6 (Somatotropin)
CN 0 (Gonadotropins)

L84 ANSWER 7 OF 8 MEDLINE
AN **79044379** MEDLINE
DN **79044379** PubMed ID: **213226**
TI Role of tissue culture in prediction of malignancy.
AU **Kornblith P L**
SO CLINICAL NEUROSURGERY, (1978) 25 346-76.
Journal code: 2985141R. ISSN: 0069-4827.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 197901
ED Entered STN: 19900314
Last Updated on STN: 19970203
Entered Medline: 19790126

AB It is now clear that tissue culture has a role to play in the prediction of the degree of malignancy of human brain tumors. The fact that virtually all human brain tumors grow at least for some interval in culture allows application of tissue culture to the study of all tumors. This almost universal culturability of tumors is truly a singular virtue of intracranial neoplasms. No other human solid tumor group has been so amenable to in vitro growth or study. Up to the present time, despite our experience with over 1100 human brain tumor cultures, we have been extremely conservative in altering patient management on the basis of in vitro data. As is now apparent, the basis for direct input into the clinical milieu exists and it is necessary to work on a patient by patient basis to see how well the existing criteria can be applied to help guide management. Indeed the emphasis given to tissue culture by Rubinstein already confirms the current interest in the applicability of tissue culture data to neuropathological study of tumors (48). Clearly for certain tumors which tend to be benign, tissue culture can serve to alert the clinician to the perhaps unexpected malignant potential of the lesion. For the malignant tumors, the degree of malignancy, the probable

biological behavior, the role of host defense factors and therapeutic agents can all be more quantitatively defined for the individual patient by the detailed study of the patient's cultured cells. With improvement in the surgical treatment of benign tumors and with better chemotherapeutic and radiotherapeutic measures for malignant neoplasms this more detailed and precise characterization of tumor behavior has become increasingly relevant to the optimization of the clinical management of the brain tumor patient.

CT Check Tags: Animal; Human; Support, U.S. Gov't, P.H.S.
Actins: ME, metabolism
Astrocytoma: UL, ultrastructure
Brain: UL, ultrastructure
Brain Neoplasms: IM, immunology
Brain Neoplasms: TH, therapy
***Brain Neoplasms: UL, ultrastructure**
Cell Communication
Cell Survival
Chromosome Aberrations
Ependymoma: UL, ultrastructure
Inclusion Bodies: UL, ultrastructure
Kinetics
Medulloblastoma: UL, ultrastructure
Meningioma: UL, ultrastructure
Mitosis
Neoplasm Invasiveness
Oligodendroglioma: UL, ultrastructure
Tissue Culture: MT, methods
CN 0 (Actins)

L84 ANSWER 8 OF 8 MEDLINE
AN **75067689** MEDLINE
DN **75067689** PubMed ID: **4439230**
TI Improved assay for cytotoxic antiglioma antibody.
AU Quindlen E A; Dohan F C Jr; **Kornblith P L**
SO SURGICAL FORUM, (1974) 25 (0) 464-6.
Journal code: 0337723. ISSN: 0071-8041.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 197503
ED Entered STN: 19900310
Last Updated on STN: 19900310
Entered Medline: 19750318
CT Check Tags: Animal; Human
*Antibodies, Neoplasm: AN, analysis
***Astrocytoma: IM, immunology**
Cytotoxicity Tests, Immunologic
Immunoassay
Rabbits
CN 0 (Antibodies, Neoplasm)

=> d his

(FILE 'HOME' ENTERED AT 09:39:27 ON 08 APR 2003)
SET COST OFF

FILE 'MEDLINE' ENTERED AT 09:39:55 ON 08 APR 2003
L1 1424959 S C4./CT
L2 169760 S TUMOR CELLS, CULTURED+NT/CT
L3 1192844 S L1/MAJ OR L2/MAJ
E CULTURE MEDIUM/CT

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                E E10+ALL
                E E2+ALL
L4          68966 S E4+NT
L5         636720 S E15+NT
L6          36910 S L4/MAJ OR L5/MAJ
L7        1215435 S L3,L6
L8        1190094 S L7 AND L1
                E DRUG SCREENING/CT
                E E4+ALL
L9          10395 S E17+NT
                E E16+ALL
L10         72779 S E12+NT
L11          6112 S L8 AND L9
L12          8256 S L8 AND L10
L13          8256 S L11,L12
L14          4127 S L13 AND D22./CT
L15          4127 S L14 AND L1-L14
L16            96 S L15 AND BIOPS?
L17             2 S L16 NOT AB/FA
L18            94 S L16 NOT L17
L19          2610 S L15 AND PY<=1996
L20            17 S L19 AND BIOPSY+NT/CT
L21            62 S L18 AND L19
L22            64 S L20,L21
L23             2 S L22 NOT AB/FA
L24            62 S L22 NOT L23
L25            51 S L24/ENG
L26            14 S L15 AND SECRET?(L)FACTOR
L27            97 S L15 AND (CELL? OR TUMOR?)(L)MARKER
L28            60 S L15 AND TUMOR(L)ANTIGEN?
L29             9 S L15 AND PLASMINOGEN?(L)ACTIVAT?
L30             8 S L15 AND (URIN? OR UROKINASE)(L)PLASMINOGEN?(L)ACTIVAT?
L31             9 S L15 AND ALPHA(L)FETOPROTEIN
L32            10 S L15 AND CARCINOEMBRYONIC(L)ANTIGEN?
L33             7 S L15 AND TRANSFORM?(L)GROWTH(L)FACTOR(L)ALPHA
L34            12 S L15 AND TRANSFORM?(L)GROWTH(L)FACTOR(L)BETA
L35             2 S L15 AND MAJOR(L)HISTOCOMPAT?(L)COMPLEX
L36           203 S L26-L35
L37            77 S L36 AND L19
L38             4 S L37 AND L18,L24
L39            73 S L37 NOT L38
L40             8 S L36 AND L18
                E KORNBLITH/AU
L41           136 S E10-E14
L42           123 S L41 AND L1-L15
                SEL DN AN 17 19 93 113
L43             4 S L42 AND E1-E12
L44            13 S L41 NOT L42
                E TUMOR ANTIGENS/CT
                E E3+ALL
L45         52163 S E2+NT
                E TUMOR MARKERS/CT
                E E7+ALL
L46         72911 S E8+NT
                E E7+ALL
                E URINARY PLASMINOGEN/CT
                E E4+ALL
L47          7724 S E34+NT
                E TRANSFORMING GROWTH FACTOR/CT
                E E4+ALL
L48          2668 S E57+NT
                E TRANSFORMING GROWTH FACTOR BETA/CT
                E E3+ALL
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L49       15475 S E81+NT
                E MAJOR HISTOCOMPATIB/CT
                E E5+ALL
L50       15830 S E21+NT
                E ALPHA FETOPROTEIN/CT
                E E4+ALL
L51       10294 S E2+NT
                E CARCINOEMBRYONIC/CT
                E E4+ALL
L52       10097 S E39+NT
                E SECRETED FACTOR/CT
                E SECRET/CT
L53       44516 S SECRET?(S)FACTOR
L54      176472 S L45-L53
L55      108418 S L54 AND PY<=1996
L56       45839 S L55 AND L3
L57        1270 S L55 AND L5/MAJ
L58          23 S L56,L57 AND L4/MAJ
L59          19 S L57 AND L9,L10
L60          19 S L57 AND D22./CT
L61          54 S L58-L60
L62          10 S L61 NOT AB/FA
                E TISSUE CULTURE/CT
                E E3+ALL
L63       68966 S E4+NT
L64        1158 S L63 AND L55
L65           8 S L64 AND L9,L10
L66          10 S L64 AND D22./CT
L67          41 S L64 AND L5/MAJ
L68          98 S L61,L65-L67
L69          20 S L68 NOT AB/FA
                SEL DN AN 2
L70           1 S L69 AND E1-E3
L71          78 S L68 NOT L69
L72           5 S L43,L70
L73        2387 S L57,L64 AND PY<=1996
L74          22 S L73 AND L9
L75          28 S L73 AND D22./CT
L76          46 S L74,L75
                SEL DN AN L16 1
L77           1 S E4-E6
L78           6 S L72,L77 AND L1-L77
L79          48 S L63/MAJ AND L73
L80       10526 S L63/MAJ AND L7
L81        8083 S L80 AND PY<=1996
L82          48 S L81 AND L54
                SEL DN AN 31 40
L83           2 S E7-E12
L84           8 S L78,L83 AND L1-L83

     FILE 'MEDLINE' ENTERED AT 10:41:56 ON 08 APR 2003

     FILE 'WPIX' ENTERED AT 10:42:11 ON 08 APR 2003
L85         568 S C12Q001-24/IC,ICM,ICS
L86        1359 S G01N033-567/IC,ICM,ICS
L87        1911 S L85,L86
L88          47 S A61P035/IC,ICM,ICS,ICA,ICI AND L87
L89         307 S P63?/M0,M1,M2,M3,M4,M5,M6 AND L87
L90         281 S (B14-H? OR C14-H? OR B12-G07 OR C12-G07 OR B12-G05 OR C12-G05
L91         365 S L88-L90
L92         172 S L91 AND (PY<=1996 OR PRY<=1996 OR AY<=1996)
L93          30 S L92 AND A01N/IC,ICM,ICS
L94         158 S L92 AND G01N/IC,ICM,ICS
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L95 92 S L94 AND C12Q/IC,ICM,ICS
L96 17 S L93 AND L95
L97 57 S C12N/IC,ICM,ICS AND L95
L98 13 S L97 AND L96
L99 25 S A61K045/IC,ICM,ICS AND L92

WEST

End of Result Set

☐ Generate Collection Print

L2: Entry 2 of 2 File: DWPI Dec 13, 2001

DERWENT-ACC-NO: 2002-097070
DERWENT-WEEK: 200253

TITLE: Identifying and monitoring progress of malignancy, useful e.g. for selection of therapy, by testing monolayer cultures grown from cohesive multicellular particulates

INVENTOR: KORNBLITH, P L

PATENT-ASSIGNEE:

ASSIGNEE	CODE
KORNBLITH P L	KORNI
PRECISION THERAPEUTICS INC	PRECN

PRIORITY-DATA: 1998US-0189310 (November 10, 1998), 1996US-0679056 (July 12, 1996), 1998US-0039957 (March 16, 1998), 1998US-0095993 (June 11, 1998)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
US 20010051353 A1	December 13, 2001		024	C12Q001/02
US 6416967 B2	July 9, 2002		000	C12Q001/02

APPLICATION-DATA:

PUB-NO	APPL-DATE	APPL-NO	DESCRIPTOR
US20010051353A1	July 12, 1996	1996US-0679056	CIP of
US20010051353A1	March 16, 1998	1998US-0039957	CIP of
US20010051353A1	June 11, 1998	1998US-0095993	CIP of
US20010051353A1	November 10, 1998	1998US-0189310	
US20010051353A1		US 5728541	CIP of
US 6416967B2	July 12, 1996	1996US-0679056	CIP of
US 6416967B2	March 16, 1998	1998US-0039957	CIP of
US 6416967B2	June 11, 1998	1998US-0095993	CIP of
US 6416967B2	November 10, 1998	1998US-0189310	
US 6416967B2		US 5728541	CIP of

INT-CL (IPC): C12 Q 1/02; C12 Q 1/18; C12 Q 1/24

RELATED-ACC-NO: 1998-110245

ABSTRACTED-PUB-NO: US 6416967B
BASIC-ABSTRACT:

NOVELTY - Identifying and monitoring (M1) progress of a patient with a malignancy by:

(i) separating a sample of tumor cells into cohesive multicellular particulates (A);

(ii) growing a tissue culture monolayer from (A) to form a prime culture; and

(iii) monitoring this monolayer over time.

DETAILED DESCRIPTION - An INDEPENDENT CLAIM is also included for treating a patient with a malignancy by using (M1) to analyze malignancy-associated markers, and using results of the analysis to determine a treatment regime.

ACTIVITY - Cytostatic. No biodata is given in the source document.

MECHANISM OF ACTION - None given in the source document.

USE - The method is used to identify a malignancy, initially, to analyze cellular and secreted markers (including those indicative of complications, aggressiveness and invasiveness), to study its growth rate and the effects of treatments with various chemotherapeutic agents, including an assessment of side-effects on normal cells, also to select the best therapy in individual cases.

ADVANTAGE - The method can be used throughout the period of the malignancy and is suitable for automation. Growing (A), rather than a suspension culture, overcomes problems of overgrowth by fibroblasts.
ABSTRACTED-PUB-NO:

US20010051353A
EQUIVALENT-ABSTRACTS:

NOVELTY - Identifying and monitoring (M1) progress of a patient with a malignancy by:

(i) separating a sample of tumor cells into cohesive multicellular particulates (A);

(ii) growing a tissue culture monolayer from (A) to form a prime culture; and

(iii) monitoring this monolayer over time.

DETAILED DESCRIPTION - An INDEPENDENT CLAIM is also included for treating a patient with a malignancy by using (M1) to analyze malignancy-associated markers, and using results of the analysis to determine a treatment regime.

ACTIVITY - Cytostatic. No biodata is given in the source document.

MECHANISM OF ACTION - None given in the source document.

USE - The method is used to identify a malignancy, initially, to analyze cellular and secreted markers (including those indicative of complications, aggressiveness and invasiveness), to study its growth rate and the effects of treatments with various chemotherapeutic agents, including an assessment of side-effects on normal cells, also to select the best therapy in individual cases.

ADVANTAGE - The method can be used throughout the period of the malignancy and is suitable for automation. Growing (A), rather than a suspension culture, overcomes problems of overgrowth by fibroblasts.

CHOSEN-DRAWING: Dwg.0/9

TITLE-TERMS: IDENTIFY MONITOR PROGRESS MALIGNANT USEFUL SELECT THERAPEUTIC TEST MONOLAYER CULTURE GROW COHERE MULTICELL PARTICLE

DERWENT-CLASS: B04 D16

CPI-CODES: B04-E03G; B04-F02; B04-F02A; B04-K01L; B11-C08E1; B11-C08E3; B12-K04A1; B14-H01; D05-H08; D05-H09;

CHEMICAL-CODES:

Chemical Indexing M1 *01*

Fragmentation Code
M423 M750 M905 N102 N136 N161 Q233
Specfic Compounds
A00GTK A00GTA

Chemical Indexing M1 *02*
Fragmentation Code
M423 M750 M781 M905 N102 N136 P831 Q233 Q505
Specfic Compounds
A00H3K A00H3A A00H3D

Chemical Indexing M1 *03*
Fragmentation Code
M423 M750 M781 M905 N102 N136 P831 Q233 Q505
Specfic Compounds
A012PK A012PA A012PD

Chemical Indexing M1 *04*
Fragmentation Code
M423 M750 M781 M905 N102 N136 P831 Q233 Q505
Specfic Compounds
A00NSK A00NSA A00NSD

Chemical Indexing M6 *05*
Fragmentation Code
M905 P633 P831 Q233 Q505 R511 R515 R521 R528 R614
R637 R639

SECONDARY-ACC-NO:
CPI Secondary Accession Numbers: C2002-030166

WEST

End of Result Set

Generate Collection Print

L1: Entry 1 of 1 File: DWPI Dec 19, 2002

DERWENT-ACC-NO: 1998-110245
DERWENT-WEEK: 200303

TITLE: Assessing chemo-sensitivity of patient cells using mono-layers grown from multicellular particulates - providing samples that relate better to in vivo behaviour, particularly used to select best agents for treating tumours in individual patients

INVENTOR: KORNBLITH, P L

PATENT-ASSIGNEE:

ASSIGNEE	CODE
PRECISION THERAPEUTICS INC	PRECN

PRIORITY-DATA: 1996US-0679056 (July 12, 1996), 1998US-0040161 (March 17, 1998), 2002US-0205887 (July 26, 2002)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
US 20020192638 A1	December 19, 2002		000	C12Q001/00
WO 9802038 A1	January 22, 1998	E	015	A01N001/02
US 5728541 A	March 17, 1998		005	C12Q001/02
AU 9736493 A	February 9, 1998		000	A01N001/02
EP 912085 A1	May 6, 1999	E	000	A01N001/02
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CN 1275885 A	December 6, 2000		000	A01N001/02
IL 127964 A	July 24, 2001		000	A01N001/02
JP 2002501609 W	January 15, 2002		014	G01N033/15

DESIGNATED-STATES: AL AM AT AU AZ BA BB BG BR BY CA CH CN CU CZ DE DK EE ES FI GB GE GH HU IL IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MD MG MK MN MW MX NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR TT UA UG UZ VN YU ZW AT BE CH DE DK EA ES FI FR GB GH GR IE IT KE LS LU MC MW NL OA PT SD SE SZ UG ZW AT BE CH DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

APPLICATION-DATA:

PUB-NO	APPL-DATE	APPL-NO	DESCRIPTOR
US20020192638A1	July 12, 1996	1996US-0679056	Cont of
US20020192638A1	March 17, 1998	1998US-0040161	Cont of
US20020192638A1	July 26, 2002	2002US-0205887	
US20020192638A1		US 5728541	Cont of
WO 9802038A1	July 10, 1997	1997WO-US11595	
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AU 9736493A	July 10, 1997	1997AU-0036493	
AU 9736493A		WO 9802038	Based on
EP 912085A1	July 10, 1997	1997EP-0933267	
EP 912085A1	July 10, 1997	1997WO-US11595	
EP 912085A1		WO 9802038	Based on
AU 712302B	July 10, 1997	1997AU-0036493	
AU 712302B		AU 9736493	Previous Publ.
AU 712302B		WO 9802038	Based on
BR 9710348A	July 10, 1997	1997BR-0010348	
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CN 1275885A	July 10, 1997	1997CN-0196143	
IL 127964A	July 10, 1997	1997IL-0127964	
JP2002501609W	July 10, 1997	1997WO-US11595	
JP2002501609W	July 10, 1997	1998JP-0506069	
JP2002501609W		WO 9802038	Based on

INT-CL (IPC): A01 N 1/02; A61 K 45/00; A61 P 17/02; A61 P 31/00; A61 P 35/00; A61 P 37/02; C12 N 1/02; C12 N 5/00; C12 N 5/10; C12 Q 1/00; C12 Q 1/02; C12 Q 1/18; C12 Q 1/24; G01 N 33/15; G01 N 33/48; G01 N 33/50; G01 N 33/567

RELATED-ACC-NO: 2002-097070

ABSTRACTED-PUB-NO: US 5728541A
BASIC-ABSTRACT:

Chemosensitivity of patient cells is assessed by:

(a) separating a specimen of tissue, cell ascites or effusion fluid into multicellular particulates (MP);

(b) growing a tissue culture monolayer from cohesive MP;

(c) inoculating cells from this layer into many separate sites;

(d) treating the sites with test agents, and

(e) assessing chemosensitivity of the treated cells.

USE - The method is used to identify the best treatment agent and concentration for a particular patient, especially for treatment of cancer or other hyper-proliferative diseases such as psoriasis and for wound healing. Formation and blocking of enzymes, neurotransmitters and other biologically active compounds can also be screened for.

ADVANTAGE - By using MP, rather than enzyme-dissociated suspensions, to produce a monolayer, preparation is simplified and a cell culture that retains in vivo reactivity is formed.

Particularly, growth of malignant cells is optimised, without overgrowth of fibroblasts or other cells as often occurs in suspension cultures. The monolayers can be grown in a few weeks, contrast longer times required with single cell progeny by dilution cloning.

ABSTRACTED-PUB-NO:

WO 9802038A
EQUIVALENT-ABSTRACTS:

Chemosensitivity of patient cells is assessed by:

(a) separating a specimen of tissue, cell ascites or effusion fluid into multicellular particulates (MP);

(b) growing a tissue culture monolayer from cohesive MP;

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(d) treating the sites with test agents, and

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CHOSEN-DRAWING: Dwg.0/0 Dwg.0/0

TITLE-TERMS: ASSESS CHEMICO SENSITIVE PATIENT CELL MONO LAYER GROW MULTICELL PARTICLE SAMPLE RELATED VIVO BEHAVE SELECT AGENT TREAT TUMOUR INDIVIDUAL PATIENT

DERWENT-CLASS: B04 D16

CPI-CODES: B04-F02; B11-C08E; B12-K04A; D05-H09;

CHEMICAL-CODES:

Chemical Indexing M1 *01*
Fragmentation Code
M423 M750 M903 N102 N136 P633 P942 P943 Q233 V754

Chemical Indexing M6 *02*
Fragmentation Code
M903 P633 P831 P942 P943 Q233 R515 R521 R627 R639

SECONDARY-ACC-NO:
CPI Secondary Accession Numbers: C1998-036204

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L3: Entry 3 of 5 File: USPT Mar 17, 1998

US-PAT-NO: 5728541
DOCUMENT-IDENTIFIER: US 5728541 A

TITLE: Method for preparing cell cultures from biologial specimens for chemotherapeutic and other assays

DATE-ISSUED: March 17, 1998

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kornblith; Paul L.	Pittsburgh	PA		

ASSIGNEE-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY	TYPE CODE
Precision Therapeutics, Inc.	Pittsburgh	PA			02

APPL-NO: 08/ 679056 [PALM]
DATE FILED: July 12, 1996

INT-CL: [06] C12 Q 1/02, C12 Q 1/18

US-CL-ISSUED: 435/29; 435/30, 435/32, 435/261
US-CL-CURRENT: 435/29; 435/261, 435/30, 435/32

FIELD-OF-SEARCH: 435/2, 435/29, 435/30, 435/32, 435/240.2, 435/261, 435/803

PRIOR-ART-DISCLOSED:

U.S. PATENT DOCUMENTS

Search Selected Search ALL

	PAT-NO	ISSUE-DATE	PATENTEE-NAME	US-CL
☐	4423145	December 1983	Stampfer et al.	435/32
☐	4937187	June 1990	Rotman	435/30
☐	5242806	September 1993	Yen-Maguire et al.	435/32
☐	5270172	December 1993	Morgan	435/29
☐	5443950	August 1995	Naughton et al.	435/1

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Cancer Research vol. 55; Julia T. Arnold. Betty P. Wilkinson. Sheela Sharma, and Vernon E. Steele; `Evaluation of Chemopreventive Agents in Different Mechanistic Classes Using a Rat Tracheal Epithelial Cell Culture Transformation Assay.sup.1 `; Feb. 1, 1995; pp. 537-543.
Anticancer Research vol. 13; Anna Kruczynski and Robert Kiss; `Evidence of a Direct Relationship Between the Increase in the in Vitro Passage Number of Human Non-Small-Cell-Lung cancer Primocultures and their Chemosensitivity`; 1993; pp. 507-514.

ART-UNIT: 121

PRIMARY-EXAMINER: Gitomer; Ralph

ABSTRACT:

An improved system for screening a multiple of candidate therapeutic or chemotherapeutic agents for efficacy as to a specific patient, in which a tissue sample from the patient is harvested, cultured and separately exposed to a plurality of treatments and/or therapeutic agents for the purpose of objectively identifying the best treatment or agent for the particular patient. The system includes the initial preparation of cohesive multicellular particulates of the tissue sample, rather than enzymatically dissociated cell suspensions or preparations, for initial tissue culture monolayer preparation. Practical monolayers of cells may thus be formed to enable meaningful screening of a plurality of treatments and/or agents. By subjecting uniform samples of cells to a wide variety of active agents (and concentrations thereof), the most promising agent and concentration for treatment of a particular patient can be determined. For assays concerning cancer treatment, a two-stage evaluation is contemplated in which both acute cytotoxic and longer term inhibitory effect of a given anti-cancer agent are investigated.

7 Claims, 0 Drawing figures
Exemplary Claim Number: 1

BRIEF SUMMARY:

1 FIELD OF THE INVENTION

2 The invention relates to screening and testing of active agents, including chemotherapeutic agents, to predict potential efficacy in individual patients in whom treatment with such agents is indicated.

3 INTRODUCTION

4 All active agents including chemotherapeutic active agents are subjected to rigorous testing as to efficacy and safety prior to approval for medical use in the United States. Methods of assessing efficacy have included elaborate investigations of large populations in double blind studies as to a given treatment method and/or active agent, with concommitant statistical interpretation of the resulting data, but these conclusions are inevitably generalized as to patient populations taken as a whole. In many pharmaceutical disciplines and particularly in the area of chemotherapy, however, the results of individual patient therapy may not comport with generalized data--to the detriment of the individual patient. The need has been long recognized for a method of assessing the therapeutic potential of active agents, including but not limited to chemotherapeutic agents, for their efficacy as to a given individual patient, prior to the treatment of that patient.

5 Prior art assays already exist which expose malignant tissue of various types to a plurality of active agents, for the purpose of assessing the best choice for therapeutic administration. For example, in Kruczynski, A., et al., "Evidence of a direct relationship between the increase in the in vitro passage number of human non-small-cell-lung cancer primocultures and their chemosensitivity," Anticancer Research, vol. 13, no. 2, pp. 507-513 (1993), chemosensitivity of non-small-cell-lung cancers was investigated in in vivo grafts, in in vitro primocultures and in commercially available long-term cancer cell lines. The increase in chemosensitivity was documented and correlated with morphological changes in the cells in question. Sometimes animal model malignant cells and/or established cell cultures are tested with prospective therapy agents, see for example Arnold, J. T., "Evaluation of chemopreventive agents in different mechanistic classes using a rat tracheal epithelial cell culture transformation assay," Cancer Res., vol. 55, no. 3, pp. 537-543 (1995).

6 When actual patient cells are used to form in vitro assays focussed on individual patients, in typical prior art processes the cells are harvested (biopsied) and trypsinized (connective tissue digested with the enzyme trypsin) to yield a cell suspension suitable for conversion to the desired tissue culture form. The in vitro tissue culture cell collections which result from these techniques are generally plagued by their inability accurately to imitate the chemosensitivity of the original tumor or other cell biopsy. Standard cloning and tissue culture techniques are moreover excessively complicated and expensive for use in a patient-by-patient assay setting. A need thus remains for a technique of tissue culture preparation which provides cell cultures, for drug screening purposes, in which after simple preparation the cell cultures react in a manner equivalent to their in vivo reactivity, to enable drug or chemotherapeutic agent screening as to a particular patient for whom such screening is indicated.

7 SUMMARY OF THE INVENTION

8 In order to meet this need, the present invention is an improved system for screening a multiple of candidate therapeutic or chemotherapeutic agents for efficacy as to a specific patient, in which a tissue sample from the patient is harvested, cultured and separately exposed to a plurality of treatments and/or therapeutic agents for the purpose of objectively identifying the best treatment for the cultured cells obtained from the patient. Specific method innovations such as tissue sample preparation techniques render this method practically as well as theoretically useful. One particularly important tissue sample preparation technique is the initial preparation of cohesive multicellular particulates of the tissue sample, rather than enzymatically dissociated cell suspensions or preparations, for initial tissue culture monolayer preparation. With respect to the culturing of malignant cells, for example, it is believed (without any intention of being bound by the theory) that by maintaining the malignant cells within a multicellular particulate of the originating tissue, growth of the malignant cells themselves is facilitated versus the overgrowth of fibroblasts or other cells which tends to occur when suspended tumor cells are grown in culture. Practical monolayers of cells may thus be formed to enable meaningful screening of a plurality of treatments and/or agents. Growth of cells is monitored to ascertain the time to initiate the assay and to determine the growth rate of the cultured cells; sequence and timing of drug addition is also monitored and optimized. By subjecting uniform samples of cells to a wide variety of active agents (and concentrations thereof), the most efficacious agent can be determined. For assays concerning cancer treatment, a two-stage evaluation is contemplated in which both acute cytotoxic and longer term inhibitory effect of a given anti-cancer agent are investigated.

DETAILED DESCRIPTION:

1 DETAILED DESCRIPTION OF THE INVENTION

2 The present invention is a system for screening a multiple of candidate therapeutic or chemotherapeutic agents for efficacy as to a specific patient, in which a tissue sample from the patient is harvested and separately exposed to a plurality of treatments and/or therapeutic agents for the purpose of objectively identifying the best treatment or agent. Specific method innovations such as tissue sample preparation techniques render this method practically as well as theoretically useful. One particularly important tissue sample preparation technique is the initial preparation of cohesive multicellular particulates (explants) of the tissue sample, rather than enzymatically dissociated cell suspensions or preparations, for initial tissue culture monolayer preparation. Cell growth, and sequence and timing of drug addition, are monitored and optimized.

3 An important application of the present invention is the screening of chemotherapeutic agents and other antineoplastic therapies against tissue culture preparations of malignant cells from the patients from whom malignant samples are biopsied. Related anti-cancer therapies which can be screened using the inventive system are both radiation therapy and agents which enhance the cytotoxicity of radiation, as well as immunotherapeutic anti-cancer agents. Screening processes for treatments or therapeutic agents for nonmalignant syndromes are also embraced within this invention, however, and include without limitation agents which combat hyperproliferative syndromes, such as psoriasis, or wound healing agents. Nor is the present efficacy assay limited only to the screening of active agents which speed up (healing) or slow down (anti-cancer, anti-hyperproliferative) cell growth because agents intended to enhance or to subdue intracellular biochemical functions may be tested in the present tissue culture system also. For example, the formation or blocking of enzymes, neurotransmitters and other biochemicals may be screened with the present assay methods prior to treatment of the patient.

4 When the patient is to be treated for the presence of tumor, in the preferred embodiment of the present invention a tumor biopsy of >100 mg of non-necrotic, non-contaminated tissue is harvested from the patient by any suitable biopsy or surgical procedure known in the art. Biopsy sample preparation generally proceeds as follows under a Laminar Flow Hood which should be turned on at least 20 minutes before use. Reagent grade ethanol is used to wipe down the surface of the hood prior to beginning the sample preparation. The tumor is then removed, under sterile conditions, from the shipping container and is minced with sterile scissors. If the specimen arrives already minced, the individual tumor pieces should be divided into four groups. Using sterile forceps, each undivided tissue quarter is then placed in 3 ml sterile growth medium (Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin) and systematically minced by using two sterile scalpels in a scissor-like motion, or mechanically equivalent manual or automated opposing incisor blades. This cross-cutting motion is important because the technique creates smooth cut edges on the resulting tumor multicellular particulates. Preferably but not necessarily, the tumor particulates each measure 1 mm.sup.3. After each tumor quarter has been minced, the particles are plated in culture flasks using sterile pasteur pipettes (9 explants per T-25 or 20 particulates per T-75 flask). Each flask is then labelled with the patient's code, the date of explantation and any other distinguishing data. The explants should be evenly distributed across the bottom surface of the flask, with initial inverted incubation in a 37.degree. C. incubator for 5-10 minutes, followed by addition of about 5-10 ml sterile growth medium and further incubation in the normal, non-inverted position. Flasks are placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks should be checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the explants will foster growth of cells into a monolayer. With respect to the culturing of malignant cells, it is believed (without any intention of being bound by the theory) that by maintaining the malignant cells within a multicellular particulate of the originating tissue, growth of the malignant cells themselves is facilitated versus the overgrowth of fibroblasts (or other unwanted cells) which tends to occur when suspended tumor cells are grown in culture.

5 The use of the above procedure to form a cell monolayer culture maximizes the growth of malignant cells from the tissue sample, and thus optimizes ensuing tissue culture assay of chemotherapeutic action of various agents to be tested. Enhanced growth of actual malignant cells is only one aspect of the present invention, however; another important feature is the growth rate monitoring system used to oversee growth of the monolayer once formed. Once a primary culture and its derived secondary monolayer tissue culture has been initiated, the growth of the cells is monitored to ascertain the time to initiate the chemotherapy assay and to determine the growth rate of the cultured cells.

6 Monitoring of the growth of cells is conducted by counting the cells in the monolayer on a periodic basis, without killing or staining the cells and

without removing any cells from the culture flask. The counting may be done visually or by automated methods, either with or without the use of estimating techniques known in the art (counting in a representative area of a grid multiplied by number of grid areas, for example). Data from periodic counting is then used to determine growth rates which may or may not be considered parallel to growth rates of the same cells in vivo in the patient. If growth rate cycles can be documented, for example, then dosing of certain active agents can be customized for the patient. The same growth rate can be used to evaluate radiation treatment periodicity, as well. It should be noted that with the growth rate determinations conducted while the monolayers grow in their flasks, the present method requires no hemocytometry, flow cytometry or use of microscope slides and staining, with all their concommitant labor and cost.

7 Protocols for monolayer growth rate generally use a phase-contrast inverted microscope to examine culture flasks incubated in a 37.degree. C. (5% CO.sub.2) incubator. When the flask is placed under the phase-contrast inverted microscope, ten fields (areas on a grid inherent to the flask) are examined using the 10.times. objective, with the proviso that the ten fields should be non-contiguous, or significantly removed from one another, so that the ten fields are a representative sampling of the whole flask. Percentage cell occupancy for each field examined is noted, and averaging of these percentages then provides an estimate of overall percent confluency in the cell culture. When patient samples have been divided between two or among three or more flasks, an average cell count for the total patient sample should be calculated. The calculated average percent confluency should be entered into a process log to enable compilation of data--and plotting of growth curves--over time. Monolayer cultures may be photographed to document cell morphology and culture growth patterns. The applicable formula is: ##EQU1## As an example, therefore, if the estimate of area occupied by the cells is 30% and the total area of the field is 100%, percent confluency is 30/100, or 30.

8 Adaptation of the above protocol for non-tumor cells is straightforward and generally constitutes an equivalent procedure.

9 Active agent screening using the cultured cells does not proceed in the initial incubation flask, but generally proceeds using plates such as microtiter plates. The performance of the chemosensitivity assay used for screening purposes depends on the ability to deliver a reproducible cell number to each row in a plate and/or a series of plates, as well as the ability to achieve an even distribution of cells throughout a given well. The following procedure assures that cells are reproducibly transferred from flask to microtiter plates, and cells are evenly distributed across the surface of each well.

10 The first step in preparing the microtiter plates is, of course, preparing and monitoring the monolayer as described above. The following protocol is exemplary and susceptible of variation as will be apparent to one skilled in the art. Cells are removed from the culture flask and a cell pellet is prepared by centrifugation. The cell pellet derived from the monolayer is then suspended in 5 ml of the growth medium and mixed in a conical tube with a vortex for 6 to 10 seconds. The tube is then rocked back and forth 10 times. A 36 .mu.l droplet from the center of the conical tube is pipetted onto one well of a 96 well plate. A fresh pipette is then used to pipette a 36 .mu.l aliquot of trypan blue solution, which is added to the same well, and the two droplets are mixed with repeated pipette aspiration. The resulting admixture is then divided between two hemocytometer chambers for examination using a standard light microscope. Cells are counted in two out of four hemocytometer quadrants, under 10.times. magnification. Only those cells which have not taken up the trypan blue dye are counted. This process is repeated for the second counting chamber. An average cell count per chamber is thus determined. Using means known in the art, the quadrant count values are checked, logged, multiplied by 10.sup.4 to give cells/ml, and the total amount of fluid (growth medium) necessary to suspend remaining cell aliquots is calculated accordingly.

11 After the desired concentration of cells in medium has been determined,

additional cell aliquots from the monolayer are suspended in growth medium via vortex and rocking and loaded into a Terasaki dispenser known in the art. Aliquots of the prepared cell suspension are delivered into the microtiter plates using Terasaki dispenser techniques known in the art. A plurality of plates may be prepared from a single cell suspension as needed. Plates are then wrapped in sterile wet cotton gauze and incubated in an incubator box by means known in the art.

12 After the microtiter plates have been prepared, exposure of the cells therein to active agent is conducted according to the following exemplary protocol. During this portion of the inventive assay, the appropriate amount of specific active agent is tranferred into the microtiter plates prepared as described above. A general protocol, which may be adapted, follows. Each microtiter plate is unwrapped from its wet cotton gauze sponge and microscopically examined for cell adhesion. Control solution is dispensed into delineated rows of wells within the grid in the microtiter plate, and appropriate aliquots of active agent to be tested are added to the remaining wells in the remaining rows. Ordinarily, sequentially increasing concentrations of the active agent being tested are administered into progressively higher numbered rows in the plate. The plates are then rewrapped in their gauze and incubated in an incubator box at 37.degree. C. under 5% CO.sub.2. After a predefined exposure time, the plates are unwrapped, blotted with sterile gauze to remove the agent, washed with Hank's Balance Salt Solution, flooded with growth medium, and replaced in the incubator in an incubator box for a predefined time period, after which the plates may be fixed and stained for evaluation.

13 Fixing and staining may be conducted according to a number of suitable procedures; the following is representative. After removal of the plates from the incubator box, culture medium is poured off and the plates are flooded with Hank's Balance Salt Solution. After repeated flooding (with agitation each time) the plates are then flooded with reagent grade ethanol for 2-5 minutes. The ethanol is then poured off. Staining is accomplished with approximately 5 ml of Giemsa Stain per plate, although volume is not critical and flooding is the goal. Giemsa stain should be left in place 5 min. .+-.30 seconds as timing influences staining intensity. The Giemsa stain is then poured off and the plates are dipped 3 times cold tap water in a beaker. The plates are then inverted, shaken vigorously, and air dried overnight (with plate lids off) on a rack on a laboratory bench. Cells per well are then counted manually or by automated and/or computerized means, to derive data regarding chemosensitivity of cells at various concentrations of exposure. One particularly useful computer operating environment for counting cells is the commercially available OPTIMATE compiler, which is designed to permit an optical counting function well suited to computerized cell counting procedures and subsequent calculations.

14 The above procedures do not change appreciably when cell growth promoters are assayed rather than cell arresting agents such as chemotherapeutic agents. The present assay allows cell death or cell growth to be monitored with equal ease. In any case, optimization of use of the present system will involve the comparative testing of a variety of candidate active agents, for selection of the best candidate for patient treatment based upon the in vitro results. One particularly advantageous embodiment of the above described invention comprises a two-stage assay for cytotoxicity followed by evaluation of longer-term inhibitory effect. Chemotherapeutic agents may thus be evaluated separately for both their direct chemotherapeutic effect as well as for their longer duration efficacy.

15 Identification of one or more active agents or chemotherapeutic agents is peripheral to the present invention, which is intended for the efficacy screening of any or all of them as to a given patient. Literally any active agent may be screened according to the present invention; listing exemplary active agents is thus omitted here.

16 The essence of the invention thus includes the important feature of the

simplicity of the present system--cohesive multicellular particulates of the patient tissue to be tested are used to form cell monolayers; growth of those monolayers is monitored for accurate prediction of correlating growth of the same cells in vivo; and differing concentrations of a number of active agents may be tested for the purpose of determining not only the most appropriate agent but the most appropriate concentration of that agent for actual patient exposure (according to the calculated cell growth rates). It is also important to note, in the context of the invention, that the present system allows in vitro tests to be conducted in suspensions of tissue culture monolayers grown in nutrient medium under fast conditions (a matter of weeks), rather than with single cell progeny produced by dilution cloning over long periods of time. In some cases, the present invention is a two stage assay for both cytotoxicity and the longer-term growth inhibitory.

17 Although the present invention has been described with respect to specific materials and methods above, the invention is only to be considered limited insofar as is set forth in the accompanying claims.

CLAIMS:

I claim:

1. A method for preparing a biopsy sample of tissue containing malignant cells for chemosensitivity testing, comprising:

(a) obtaining a tumor specimen;

(b) mechanically separating said specimen into cohesive multicellular particulates having a size of about 1 mm.sup.3 ;

(c) growing a tissue culture monolayer from said cohesive multicellular particulates;

(d) inoculating cells from said monolayer into a plurality of segregated sites;

(e) treating said plurality of sites with at least one agent;

(f) examining said plurality of sites; and

(g) assessing chemosensitivity of the cells in said plurality sites.

2. The method according to claim 1 wherein said plurality of segregated sites comprise a plate containing a plurality of wells therein.

3. The method according to claim 2 wherein said plurality of wells is treated over a length of time adequate to permit assessment of both initial cytotoxic effect and longer-term inhibitory effect of said at least one agent.

4. The method according to claim 3 wherein step (d) is accomplished by a multiple well pipetting device.

5. The method according to claim 4 wherein the cells in step (d) are further suspended in medium prior to inoculation into said plate containing a plurality of wells therein.

6. The method according to claim 5 wherein said agent is at least one of the agents selected from group consisting of a radiation therapy agent, a radiation therapy sensitizing agent and a radiation therapy desensitizing agent.

7. The method according to claim 5 wherein said agent is an immunotherapeutic agent.

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L2: Entry 1 of 2 File: USPT Jul 9, 2002

US-PAT-NO: 6416967
DOCUMENT-IDENTIFIER: US 6416967 B2

TITLE: Method of using multicellular particulates to analyze malignant or hyperproliferative tissue

DATE-ISSUED: July 9, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Kornblith; Paul L.	Pittsburgh	PA		

ASSIGNEE-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY	TYPE CODE
Precision Therapeutics, Inc.	Pittsburgh	PA			02

APPL-NO: 09/ 189310 [PALM]
DATE FILED: November 10, 1998

PARENT-CASE:
RELATED APPLICATION This is a Continuation-In-Part of U.S. application Ser. No. 08/679,056, filed Jul. 12, 1996, now U.S. Pat. No. 5,728,541, granted Mar. 17, 1998; U.S. application Ser. No. 09/095,993, filed Jun. 11, 1998; and U.S. application Ser. No. 09/039,957, filed Mar. 16, 1998.

INT-CL: [07] C12 Q 1/02

US-CL-ISSUED: 435/29; 435/30
US-CL-CURRENT: 435/29; 435/30

FIELD-OF-SEARCH: 435/29, 435/30, 435/32, 435/240.2, 435/261, 435/803

PRIOR-ART-DISCLOSED:

U.S. PATENT DOCUMENTS

Search Selected Search ALL

	PAT-NO	ISSUE-DATE	PATENTEE-NAME	US-CL
☐	4423145	December 1983	Stampfer et al.	435/32
☐	4937187	June 1990	Rotman	435/30
☐	5242806	September 1993	Yen-Maguire et al.	435/32
☐	5270172	December 1993	Morgan	435/29
☐	5607918	March 1997	Eriksson et al.	514/12
☐	5728541	March 1998	Kornblith	435/29
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"Evaluation of Chemopreventive Agents in Different Mechanistic Classes Using a Rat Tracheal Epithelial Cell Cultrue Transformation Assay." Arnold et al., Cancer Research, vol. 55, pp. 537-543 (1995).
"Evidence of a Direct Relationship Between the Increase in the In Vitro Passage Number of Human Non-Small-Cell-Lung Cancer Primocultures and Their Chemosensitivity," Kruczynski et al., Anticancer Research, vol. 13, pp. 507-514 (1993).
Arnold, J. Evaluation of Chemopreventive Agents in Different Mechanistic Classes Using a Rat Tracheal Epithelial Cell Culture Transformation Assay. Cancer Research 55:537-543, Feb. 1995.*
Kruczynski A. Evidence of a Direct Relationship Between the Increase in the in Vitro Passage Number of Human Non-Small-Cell Lung Cancer Primocultures and Their Chemosensitivity. Anticancer Research 13:507-514, 1993.

ART-UNIT: 1623

PRIMARY-EXAMINER: Gitomer; Ralph

ABSTRACT:

A comprehensive and integrated system for monitoring (identifying, tracking and analyzing) an individual patient's malignancy or hyperproliferative syndrome through the duration of a malignancy or hyperproliferative syndrome as to a specific patient is provided. Specimens of a patient's cells are collected and mechanically separated into cohesive multicellular particulates. A tissue culture monolayer is grown from the multicellular particulates to form a prime culture, and the tissue culture is monitored over a period of time. The invention allows for study of the effect of various treatment methods on cellular production of vascular endothelial growth factor.

22 Claims, 34 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 8

BRIEF SUMMARY:

1 BACKGROUND OF THE INVENTION

2 Field of the Invention

3 A system is provided for in vitro tracking of cancerous tissue over the course

of the malignancy. The system provides a method for identifying the malignancy and for determining a patient's prognosis. Further, the system provides for assessing a malignancy's invasiveness, aggressiveness, growth rate, production of extracellular markers, possible side effects and for determining the efficacy on the malignancy of a given therapeutic regimen. The system also allows for generation of a therapeutic index, which serves as an indicator of a given therapy's effectiveness against the malignancy as compared to its undesirable side effects, such as lethality to a patient's normal cells.

4 INTRODUCTION

5 Tracking a malignancy in a patient according to prior art methods is an inaccurate process which involves identification of the malignancy through techniques including biopsy and subsequent histological, biochemical, and immunochemical techniques and regularly monitoring the malignancy's progression by invasive (i.e., biopsy) or noninvasive (i.e., x-ray, nuclear imaging, Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET)) methods. These methods are often expensive, inconvenient, painful and usually involve hospital visits and safety risks. It is, therefore, desirable to reduce a patient's exposure to such methods. Furthermore, identification of a malignancy as a known variety of malignancy is often helpful in determining a suitable therapeutic approach and expected prognosis. However, even individually identifiable malignancies differ from patient-to-patient in their growth characteristics and in their responsiveness to treatment.

6 Determination of the growth rate, invasiveness and aggressiveness of a given malignancy is critical to prognosis and to the choice of therapies. A patient with a poor prognosis might be given a therapeutic regimen which might be more effective than another regimen but more risky to the patient. A patient with a better prognosis might be given a therapeutic regimen which is less aggressive and less risky to the patient, but which might not be as effective as often as a more dangerous therapy. Similarly, if a malignancy produces factors or creates conditions which cause a dangerous side effect, such as a thrombogenesis, the patient can be treated, preferably prophylactically, for the condition.

7 Current methodologies for determining growth rate, invasiveness, aggressiveness or which track the progression of a malignancy include biopsy and short-term culture, which can include drawing of blood or other bodily fluids, or semi- or non-invasive techniques such as x-ray and nuclear imaging. At any given time, a patient could be subject to multiple procedures, depending upon when the information is needed by the physician. Each procedure requires the presence of the patient and usually creates risk or pain. These procedures also can increase the stress level of the patient, which often is an exacerbating factor in cancer and associated prognoses. It is therefore, desirable to reduce the frequency of such procedures.

8 Identification of an effective therapeutic regimen is critically important to a patient. Often, once the malignancy is identified, a therapy is chosen based upon prior research on that type of malignancy and is not tailored to the sensitivities of the malignancy of a given patient. Often secondary therapies are needed because a first choice was ineffective. Valuable treatment time can be lost and a patient's life can be threatened.

9 All active agents including chemotherapeutic active agents are subjected to rigorous testing as to efficacy and safety prior to approval for medical use in the United States. Methods of assessing efficacy have included elaborate investigations of large populations in double blind studies as to a given treatment method and/or active agent, with concomitant statistical interpretation of the resulting data, but these conclusions are inevitably generalized as to patient populations taken as a whole. In many pharmaceutical disciplines and particularly in the area of chemotherapy, however, the results of individual patient therapy may not comport with generalized data--to the detriment of the individual patient. The need has been long recognized for a

method of assessing the therapeutic potential of active agents, including but not limited to chemotherapeutic agents, for their efficacy as to a given individual patient, prior to the treatment of that patient. This need also applies to assessing the therapeutic potential as to radiation therapies, combined radiation/drug therapies and cellular immunotherapies.

10 Prior art assays already exist which expose malignant tissue of various types to a plurality of active agents, for the purpose of assessing the best choice for therapeutic administration. For example, in Kruczynski, A., et al., "Evidence of a direct relationship between the increase in the in vitro passage number of human non-small-cell-lung cancer primocultures and their chemosensitivity," Anticancer Research, vol. 13, no. 2, pp. 507-513 (1993), chemosensitivity of non-small-cell-lung cancers was investigated in vivo grafts, in vitro primocultures and in commercially available long-term cancer cell lines. The increase in chemosensitivity was documented and correlated with morphological changes in the cells in question. Sometimes animal model malignant cells and/or established cell cultures are tested with prospective therapy agents, see for example Arnold, J. T., "Evaluation of chemopreventive agents in different mechanistic classes using a rat tracheal epithelial cell culture transformation assay," Cancer Res., vol. 55, no. 3, pp. 537-543 (1995).

11 In vitro prior art techniques present the further shortcoming that assayed cells do not necessarily express the cellular markers they would express in vivo. This is regrettable because the determination of expression of certain secreted or cellular markers, secreted factors or tumor antigens or lack thereof can be useful for both identification and therapeutic purposes. For instance, members of the fibrinolytic system such as urokinase-type plasminogen activator (u-PA) and plasminogen activator inhibitors type 1 (PAI-1) are up-regulated in malignant brain tumors. See, e.g., Jasti S. Rao, et al., "The Fibrinolytic System in Human Brain Tumors: Association with Pathophysiological Conditions of Malignant Brain Tumors," Advances in Neuro-Oncology II, Kornblith P L, Walker M D (eds) Futura (1997). Other secreted factors such as .alpha.-fetoprotein, carcinoembryonic antigen and transforming growth factors .alpha. and .beta. have been found to be indicative of various cancers and/or cancer progression (see also, Singhal et al., "Elevated Plasma Osteopontin in Metastatic Breast Cancer Associated with Increased Tumor Burden and Decreased Survival," Clinical Cancer Research, vol. 3, 605-611, (April 1997); Kohno et al., "Comparative Studies of CAM 123-6 and Carcinoembryonic Antigen for the Serological Detection of Pulmonary Adenocarcinoma," Cancer Detection and Prevention, 21 (2): 124-128 (1997)). These examples are but a few of the many factors that may be used to identify diseased cells.

12 Cellular markers also include metastatic markers, indicative of metastatic potential, i.e., invasiveness and aggressiveness, which is relevant to the progression of a given malignancy and to a patient's prognosis. First, markers indicating the invasiveness of a given malignancy indicate the ability of the malignancy to infiltrate and to destroy adjacent tissue. As an example, for epithelial malignancies, invasiveness markers are indicative of the ability of the malignancy to infiltrate beneath the epithelial basement membrane. Invasiveness markers can include the presence of proteolytic enzymes or angiogenic factors. A second category of metastatic marker indicates growth conditions of the malignancy. For instance, a malignancy could require for instance a prostate-specific factor for growth. Invasiveness and aggressiveness factors are often present in serum or in tissue culture media.

13 Relevant to a patient's prognosis and, incidentally, to the identification of a malignancy is the presence of markers, cellular or secreted, which lead to complications beyond those involved with uncontrolled growth and invasion by a malignancy. For instance, secretion by the malignancy of thrombogenic substances by the malignancy can result in blood clotting, resulting in thrombophlebitis or other thrombotic events such as pulmonary thrombosis. Identification of a thrombotic potential indicates treatment (preferably prophylactically) with thrombolytic substances.

14 When a specific patient's cells are used in vitro assays in typical prior art processes the cells are harvested (biopsied) and trypsinized (connective tissue digested with the enzyme trypsin) to yield a cell suspension purportedly suitable for conversion to the desired tissue culture form. The in vitro tissue culture cell collections which result from these techniques are generally plagued by their inability accurately to imitate the chemosensitivity or therapeutic sensitivity of the original tumor or other cell biopsy. These collections often do not express cellular markers in the same manner that they would in vivo. A need thus remains for a technique of tissue culture preparation which provides cell cultures, allowing identification of a malignancy, accurate tracking of the malignancy's progress in a patient and therapy screening, in which, after simple preparation, the cell cultures react in a manner equivalent to their in vivo reactivity. The culture method would enable drug or chemotherapeutic agent, radiation therapy and/or cellular immunotherapy screening as to a particular patient for whom such screening is indicated.

15 A need also remains for a technique of tissue culture preparation which provides cell cultures for screening for expressed markers or factors where the cultured cells express the markers or factors in a manner indicative of their in vivo expression of the same. A further need also remains for a tissue culture preparation which allows for morphological study of the cells. Lastly, a need remains for a tissue culture system in which progression of an individual malignancy can be studied as indicative of the in vivo progression of the malignancy.

16 SUMMARY OF THE INVENTION

17 A comprehensive and integrated unified system for monitoring (i.e., identifying, tracking and analyzing) an individual patient's malignancy through the duration of a malignancy as to a specific patient is provided. The method of the present invention allows for initial identification of a malignancy, identification of malignancy-specific cellular or secreted markers, identification of cellular or secreted markers indicative of complications, study of the invasiveness and aggressiveness of the malignancy, study of the growth rate of the malignancy, study of the effect of therapies on the malignancy as compared to control cells of the same patient (chemosensitivity versus toxicity) and the identification of a therapeutic index (i.e., the ratio of chemosensitivity:toxicity), study of tumor morphology and study of histological and cytochemical markers.

18 The method of the present invention includes the steps of collecting a tissue sample or specimen of a patient's cells and separating the specimen into cohesive multicellular particulates (explants) of the tissue sample, rather than enzymatically digested cell suspensions or preparations. The cells are then grown as a tissue culture monolayer from the multicellular particulates to form a prime culture. A specimen can be taken from a patient at any relevant site, including but not limited to tissue, ascites or effusion fluid. Samples may also be taken from body fluid or exudates, as is appropriate. A tissue culture monolayer, designated as the prime culture, can be grown in any method known in the art for growing such a monolayer, for instance in tissue culture plates or flasks. If the malignant cells originate from solid tissue, however, the tissue must be subdivided into small pieces from which a tissue culture monolayer is then grown out.

19 Once a prime culture is established from a patient's malignancy, the prime culture can be maintained without any treatments beside normal feedings and passage techniques, as indicative of the growth of the malignancy absent treatment. However, subcultures of the prime culture are prepared so that the prime culture is preferably left untreated, and the cells of the prime culture are not affected by any testing. However, either the prime culture or a subculture thereof can be propagated as a reference culture. The reference culture is a culture which is treated with therapies reflective of a patient's actual treatments. For instance, if a patient is treated with a

chemotherapeutic agent, the reference culture is treated with the same agent in the same concentration. The reference culture can be monitored genotypically or phenotypically to reflect actual progress of the malignancy in the patient. Treatment of the reference culture need not be limited to anticancer therapies, but can reflect all of a patient's treatments. For instance, thrombolytic or anti-thrombogenic treatments can be applied to the reference culture to reflect a patient's treatment. Subcultures of either the prime culture or the reference culture can be used for further analysis. Preferably, since the reference culture is indicative of the current state of a malignancy at a given time, subcultures of the reference culture are analyzed further. At various points in the passage of the control culture and the reference culture, aliquots of cells from those cultures can be stored cryogenically or otherwise.

20 The tissue sample technique of the present invention is also useful in assaying expression and/or secretion of various markers, factors or antigens present on or produced by the cultured cells. These assays can be used for diagnostic purposes for monitoring the applicability of certain candidate therapeutic or chemotherapeutic agents or for monitoring the progress of treatment of the cancer with those agents.

21 A method for identifying and monitoring progress of a malignancy in an individual patient is provided including the steps of inoculating cells from either the prime culture, the reference culture or a subculture of the prime culture or of the reference culture into a plurality of segregated sites; treating the plurality of sites with at least one treating means or therapy, followed by assessment of sensitivity of cells in the site to the treating means; collecting a specimen of a patient's non-malignant cells; separating the non-malignant cells into cohesive multicellular particles; growing a tissue culture monolayer from the multicellular particulates of non-malignant cells to form a control culture; inoculating the control culture in a plurality of non-segregated sites; treating the plurality of segregated sites of the control culture with the same treating means as the segregated sites of the prime culture or a subculture thereof, followed by assessment of the sensitivity of the segregated cells of the control culture to the treating means; and comparing the sensitivity of the segregated cells of the prime culture or a subculture thereof with the sensitivity of the segregated cells of the control culture to the treating means. The assessments described above are calculations of the percentage or fraction of cells sensitive, or insensitive, to the treatment and the method further includes the step of creating a therapeutic index of a ratio of one of the percentage of or the fraction of sensitive cells or insensitive cells in the segregated cells of the control culture to one of the percentage of or the fraction of sensitive cells or insensitive cells in the segregated cells of the prime culture or subculture thereof.

22 Lastly, a method for treating a patient having a malignancy is provided, including the steps of: analyzing a patient's cells prepared according to the above-described methods for malignancy-associated markers; determining a therapeutic regimen according to the results of the analysis; and treating a patient according to the regimen. The method can further include the step of treating one of either cells cultured as a subculture of the prime culture or cells of the prime culture according to the regimen as representative of the patient's malignancy. Lastly, the method further includes determining a therapeutic index for each treating means as described above.

23 When applicable, cultures can be grown in a readable (scannable) plate and to determine percent confluence of the cells or any other parameter which can be determined in such a manner. The scanner can be operably linked with a computer or CPU to automatically input data into the computer or CPU. The computer or CPU can be programmed to automatically calculate a therapeutic index (or other relevant indices) based upon the data provided by the scanner. Alternatively, the data can be entered manually into the programmed computer or CPU to calculate the index.

DRAWING DESCRIPTION:

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A, 1B and 1C are graphs of the growth rates of three independent cell cultures.

FIGS. 2A-2F through 5A-5F are graphs depicting the results of short-term and long-term chemotherapy assays.

FIGS. 2A-2F and 3A-3F show short-term and long-term assays for a first patient.

FIGS. 4A-4F and 5A-5F show short-term and long-term assays for a second patient.

FIGS. 6 and 7 show two radiation dose versus surviving fraction curves for two glioblastoma cell lines. Cells were irradiated in microtiter plates and assayed four days post-irradiation.

FIGS. 8A-8C are graphs of survival rates of cell cultures treated with radiation (FIG. 8A) or with radiation and Taxol (FIGS. 8B and 8C).

FIGS. 9A and 9B are graphs showing data from a series of experiments where target cells from two tumor types were exposed to Activated Natural Killer (ANK) cells.

DETAILED DESCRIPTION:

1 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

2 The present invention is an improved and unified system for monitoring the progression of an individual malignancy and for identifying cellular and secreted markers, markers indicative of certain side effects of the malignancy and for screening multiple candidate therapeutic or chemotherapeutic agents for efficacy and long term effect as to a specific patient. In the method, a tissue sample from the patient is harvested, cultured and separately exposed to a plurality of treatments and/or therapeutic agents for the purpose of objectively identifying the best treatment for the cultured cells obtained from the patient. The culture techniques of the present invention also result in a monolayer of cells that express cellular markers, secreted factors and tumor antigens in a manner representative of their expression in vivo. Specific method innovations such as tissue sample preparation techniques render this method practically as well as theoretically useful. One particularly important tissue sample preparation technique is the initial preparation of cohesive multicellular particulates of the tissue sample, rather than enzymatically dissociated cell suspensions or preparations, for initial tissue culture monolayer preparation. With respect to the culturing of malignant cells, for example, it is believed (without any intention of being bound by the theory) that by maintaining the malignant cells within a multicellular particulate of the originating tissue, growth of the malignant cells themselves is facilitated versus the overgrowth of fibroblasts or other cells which tends to occur when suspended tumor cells are grown in culture. Practical monolayers of cells may thus be formed to enable meaningful screening of a plurality of treatments and/or agents as well as meaningful identification of cellular markers. In the drug assays, growth of cells is monitored to ascertain the time to initiate the assay and to determine the growth rate of the cultured cells; sequence and timing of drug addition is also monitored and optimized. By subjecting uniform samples of cells to a wide variety of active agents (and concentrations thereof), the most efficacious agent can be determined. For assays concerning cancer treatment, a two-stage evaluation is contemplated in which both acute cytotoxic and longer term inhibitory effects of a given anti-cancer agent are investigated.

3 With regard to the identification of expressed cellular markers, secreted factors or tumor antigens, with the initial culturing of the multicellular

particulates it is believed (without any intention of being bound by the theory) that because the cells are grown under conditions closer to those found in vivo, the cells express their cellular markers, secreted factors and tumor antigens in a manner more closely resembling their expression in vivo. By assaying the culture media obtained from growing a monolayer according to the inventive method or by histochemically and/or immunohistochemically assaying the cells grown under such conditions, a more accurate profile of the cellular markers or factors is obtained.

4 Thus, a comprehensive and integrated system for identifying, tracking and analyzing an individual patient's malignancy through the duration of the malignancy and thereafter is provided. The method of the present invention allows for initial identification of a malignancy, identification of malignancy-specific cellular or secreted markers, identification of cellular or secreted markers indicative of complications, study of the invasiveness and aggressiveness of the malignancy, study of the growth rate of the malignancy, study of the effect of therapies on the malignancy as compared to control cells of the same patient (chemosensitivity and/or radiosensitivity versus toxicity) and the identification of a therapeutic index (i.e., the ratio of chemosensitivity:toxicity), study of tumor morphology and study of histological, cytochemical and immunocytochemical markers.

5 The method of the present invention includes the steps of collecting a tissue sample or specimen of a patient's cells and separating the specimen into cohesive multicellular particulates (explants) of the tissue sample, rather than enzymatically digested cell suspensions or preparations. The cells are then grown as a tissue culture monolayer from the multicellular particulates to form a prime culture. A specimen can be taken from a patient at any relevant site, including but not limited to tissue, ascites or effusion fluid. Samples may also be taken from body fluid or exudates, as is appropriate. A tissue culture monolayer can be grown in any method known in the art for growing such a monolayer, for instance in tissue culture plates or flasks.

6 Once a prime culture is established from a patient's malignancy, the prime culture can be maintained without any treatments beside normal feedings and passage techniques, as indicative of the growth of the malignancy absent treatment with a therapeutic regimen. Subcultures of the prime culture are prepared so that the cells of the prime culture are not affected by any subsequent testing or treatments. Although prime culture is preferably left untreated, either the prime culture or a subculture thereof can be propagated as a reference culture. The reference culture is a culture which is treated with therapies reflective of a patient's actual treatment regimen. For instance, if a patient is treated with a chemotherapeutic agent, the reference culture is treated with the same agent in the same concentration. The reference culture can be monitored genotypically or phenotypically to reflect actual progress of the malignancy in the patient. Treatment of the reference culture need not be limited to anticancer therapies, but can reflect all of a patient's treatments. For instance, thrombolytic or anti-thrombogenic treatments, can be applied to the reference culture to reflect a patient's treatment. Subcultures of either the prime culture or the reference culture can be used for further analysis. Preferably, since the reference culture is indicative of the current state in a patient of a malignancy, subcultures of the reference culture are analyzed. At various points in the passage of the control culture and the reference culture, aliquots of cells from those cultures can be stored cryogenically, or otherwise.

7 An important further aspect of the present invention is to provide a system for screening specific tissue samples from individual patients for expressed cellular markers, secreted factors or antigens, including tumor antigens, characteristic of the tissue sample. A tissue sample from a patient is harvested and grown in a monolayer culture as described above. Culture medium in which the cultures or subcultures thereof are assayed for the presence or absence of certain factors, such as secreted tumor antigens such as PAI-1, u-PA, cancer associated serum antigen (CASA) or carcinoembryonic antigen (CEA).

These factors may be detected through use of standard assays such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA), although other assays known to those skilled in the art may be used to detect and/or to quantify the soluble factors. The cell cultures grown in this manner may also be assayed histochemically and or immunohistochemically for identification or quantification of cellular or membrane-bound markers. Examples of such markers include, without limitation, CEA, tissue polypeptide specific antigen, (TPS) and mucin antigens, such as CA 15-3, CA 549, CA 27.29 and MCA. By screening tissue samples in this manner, for production of such factors, markers or antigens, the cultured cells may be further identified, aiding the physician in treatment strategies and as a prognosis indicator. Furthermore, by combining the use of the culture technique with assaying for such markers, factors and antigens, a treatment strategy for a disease state may be optimized and treatment progression may be monitored.

8 One important aspect of analyzing tissue culture medium is that complications of a malignancy can be predicted. For instance, one common complication is thrombogenesis. A propensity towards blood clot formation can be detected in tissue culture medium by identifying thrombogenic or procoagulant factors such as, without limitation, the Lewis Y antigen (Ley), HLA-DR and other tumor procoagulants, such as cancer procoagulant (CP) and tissue factor (TF). By identifying production of thrombogenic factors, a physician can prescribe drug and/or exercise regimens, as appropriate, to prevent life and/or limb-threatening clotting.

9 Cells and/or tissue culture media from any of the prime culture, the reference culture or subcultures thereof can be analyzed for tumor aggressiveness and invasiveness markers. Presence of these markers or absence thereof is highly relevant to a patient's prognosis. Furthermore, the effect of a given therapy on any of these markers can be analyzed. For instance, a tumor may produce angiogenic factors, such as, without limitation, vascular.endothelial growth factor (VEGF), which would lead a doctor to give a patient a less favorable prognosis. Other markers can include, without limitation, factors which allow cancer cells to affix to organs other than those from which the cancer cells derive, for instance, beta 3 integrin, which participates in the ability of melanoma cells to adhere to blood vessel walls. However, the effectiveness of therapies can be assessed if the presence of the angiogenic marker is analyzed in segregated sites according to the method of the present invention. A physician can suppress a malignancy by preventing expression of factors or markers which cause the malignancy's aggressiveness or invasiveness.

10 An important application of the present invention is the screening of chemotherapeutic agents and other antineoplastic therapies in tissue culture preparations of malignant cells from the patients from whom malignant samples are biopsied. Related anti-cancer therapies which also can be screened using the inventive system include radiation therapy and agents which enhance the cytotoxicity of radiation, as well as immunotherapeutic anti-cancer agents. Screening processes for treatments or therapeutic agents for nonmalignant syndromes are also embraced within this invention and include without limitation agents which combat hyper-proliferative syndromes, such as psoriasis, or wound healing agents. Nor is the present efficacy assay limited only to the screening of active agents which speed up (healing) or slow down (anti-cancer, anti-hyper-proliferative) cell growth because agents intended to enhance or to subdue intracellular biochemical functions may be tested in the present tissue culture system also. For example, the formation or blocking of enzymes, neurotransmitters and other biochemicals may be screened with the present assay methods prior to treatment of the patient.

11 When a patient is to be treated for the presence of tumor, in the preferred embodiment of the present invention a tumor biopsy of >100 mg of non-necrotic, non-contaminated tissue is harvested from the patient by any suitable biopsy or surgical procedure known in the art. Biopsy sample preparation generally proceeds as follows under a Laminar Flow Hood which should be turned on at least 20 minutes before use. Reagent grade ethanol is used to wipe down the

surface of the hood prior to beginning the sample preparation. The tumor is then removed, under sterile conditions, from the shipping container and is minced with sterile scissors. If the specimen arrives already minced, the individual tumor pieces should be divided into four groups. Using sterile forceps, each undivided tissue quarter is then placed in 3 ml sterile growth medium (Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin) and systematically minced by using two sterile scalpels in a scissor-like motion, or mechanically equivalent manual or automated opposing incisor blades. This cross-cutting motion is important because the technique creates smooth cut edges on the resulting tumor multicellular particulates. Preferably but not necessarily, the tumor particulates each measure 1 mm.sup.3. After each tumor quarter has been minced, the particles are plated in culture flasks using sterile pasteur pipettes (9 explants per T-25 or 20 particulates per T-75 flask). Each flask is then labeled with the patient's code, the date of explanation and any other distinguishing data. The explants should be evenly distributed across the bottom surface of the flask, with initial inverted incubation in a 37.degree. C. incubator for 5-10 minutes, followed by addition of about 5-10 ml sterile growth medium and further incubation in the normal, non-inverted position. Flasks are placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks should be checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the explants will foster growth of cells into a monolayer.

12 With respect to the culturing of malignant cells, it is believed (without any intention of being bound by the theory) that by maintaining the malignant cells within a multicellular particulate of the originating tissue, growth of the malignant cells themselves is facilitated versus the overgrowth of fibroblasts (or other unwanted normal cells) which tends to occur when suspended tumor cells are grown in culture.

13 The use of the above procedure to form a cell monolayer culture maximizes the growth of malignant cells from the tissue sample, and thus optimizes ensuing tissue culture assay of chemotherapeutic action of various agents to be tested. Enhanced growth of actual malignant cells is only one aspect of the present invention; however, another important feature is the growth rate monitoring system used to oversee growth of the monolayer once formed. Once a primary culture and its derived secondary monolayer tissue culture has been initiated, the growth of the cells is monitored to ascertain the time to initiate the chemotherapy assay and to determine the growth rate of the cultured cells.

14 Monitoring of the growth of cells is conducted by counting the cells in the monolayer on a periodic basis, without killing or staining the cells and without removing any cells from the culture flask. The counting may be done visually or by automated methods, either with or without the use of estimating techniques known in the art (counting in a representative area of a grid multiplied by number of grid areas, for example). Data from periodic counting is then used to determine growth rates which may or may not be considered parallel to growth rates of the same cells in vivo in the patient. If growth rate cycles can be documented, for example, then dosing of certain active agents can be customized for the patient. The same growth rate can be used to evaluate radiation treatment periodicity, as well. It should be noted that with the growth rate determinations conducted while the monolayers grow in their flasks, the present method requires no hemocytometry, flow cytometry or use of microscope slides and staining, with all their concomitant labor and cost.

15 Protocols for monolayer growth rate generally use a phase-contrast inverted microscope to examine culture flasks incubated in a 37.degree. C. (5% CO.sub.2) incubator. When the flask is placed under the phase-contrast inverted microscope, ten fields (areas on a grid inherent to the flask) are examined using the 10.times. objective, with the proviso that the ten fields should be non-contiguous, or significantly removed from one another, so that the ten fields are a representative sampling of the whole flask. Percentage cell occupancy for each field examined is noted, and averaging of these percentages

then provides an estimate of overall percent confluency in the cell culture. When patient samples have been divided between two or among three or more flasks, an average cell count for the total patient sample should be calculated. The calculated average percent confluency should be entered into a process log to enable compilation of data--and plotting of growth curves--over time. Monolayer cultures may be photographed to document cell morphology and culture growth patterns.

16 The applicable formula is: ##EQU1##

17 As an example, therefore, if the estimate of area occupied by the cells is 30% and the total area of the field is 100%, percent confluency is 30/100, or 30.

18 Adaptation of the above protocol for non-tumor cells is straightforward and generally constitutes an equivalent procedure.

19 Active agent and/or radiation therapy screening using the cultured cells proceeds with subcultures of the prime culture or, preferably, of the reference culture. The screening can be carried out in an incubation flask, but generally proceeds using plates such as microtiter plates. In a chemotherapy/radiotherapy assay, it is desirable to grow a control culture of a patient's cells in a culture parallel to the reference or prime culture. The control culture can be grown from skin cells, as an easy source of non-malignant cells, from the same organ from which the malignant cells are derived, or from other sources, so long as the cells are typical of non-malignant cells of the patient.

20 The performance of the chemosensitivity/radiosensitivity assay used for screening purposes depends on the ability to deliver a reproducible cell number to each row in a plate and/or a series of plates, as well as the ability to achieve an even distribution of cells throughout a given well. The following procedure assures that cells are reproducibly transferred from flask to microtiter plates, and cells are evenly distributed across the surface of each well.

21 The first step in preparing the microtiter plates is, of course, preparing and monitoring the monolayer as described above. The following protocol is exemplary and susceptible of variation as will be apparent to one skilled in the art. Cells are removed from the culture flask and a cell pellet is prepared by centrifugation. The cell pellet derived from the monolayer is then suspended in 5 ml of the growth medium and mixed in a conical tube with a vortex for 6 to 10 seconds. The tube is then rocked back and forth 10 times. A 36 .mu.l droplet from the center of the conical tube is pipetted onto one well of a 96 well plate. A fresh pipette is then used to pipette a 36 .mu.l aliquot of trypan blue solution, which is added to the same well, and the two droplets are mixed with repeated pipette aspiration. The resulting admixture is then divided between two hemocytometer chambers for examination using a standard light microscope. Cells are counted in two out of four hemocytometer quadrants, under 10.times. magnification. Only those cells which have not taken up the trypan blue dye are counted. This process is repeated for the second counting chamber. An average cell count per chamber is thus determined. Using means known in the art, the quadrant count values are checked, logged, multiplied by 10.sup.4 to give cells/ml, and the total amount of fluid (growth medium) necessary to suspend remaining cell aliquots is calculated accordingly.

22 After the desired concentration of cells in medium has been determined, additional cell aliquots from the monolayer are suspended in growth medium via vortex and rocking and loaded into a Terasaki dispenser known in the art. Aliquots of the prepared cell suspension are delivered into the microtiter plates using Terasaki dispenser techniques known in the art. A plurality of plates may be prepared from a single cell suspension as needed. Plates are then wrapped in sterile wet cotton gauze and incubated in an incubator box by means known in the art.

23 After the microtiter plates have been prepared, exposure of the cells therein

to active agent and/or radiation is conducted according to the following exemplary protocol. During this portion of the inventive assay, the appropriate amount of specific active agent is transferred into the microtiter plates prepared as described above. A general protocol, which may be adapted, follows. Each microtiter plate is unwrapped from its wet cotton gauze sponge and microscopically examined for cell adhesion. Control solution is dispensed into delineated rows of wells within the grid in the microtiter plate, and appropriate aliquots of active agent to be tested are added to the remaining wells in the remaining rows. Ordinarily, sequentially increasing concentrations of the active agent or higher doses of radiation being tested are administered into progressively higher numbered rows in the plate. The plates are then rewrapped in their gauze and incubated in an incubator box at 37.degree. C. under 5% CO.sub.2. After a predefined exposure time, the plates are unwrapped, blotted with sterile gauze to remove the agent, washed with Hank's Balance Salt Solution, flooded with growth medium, and replaced in the incubator in an incubator box for a predefined time period, after which the plates may be fixed and stained for evaluation.

24 Fixing and staining may be conducted according to a number of suitable procedures; the following is representative. After removal of the plates from the incubator box, culture medium is poured off and the plates are flooded with Hank's Balance Salt Solution. After repeated flooding (with agitation each time) the plates are then flooded with reagent grade ethanol for 2-5 minutes. The ethanol is then poured off. Staining is accomplished with approximately 5 ml of Giemsa Stain per plate, although volume is not critical and flooding is the goal. Giemsa stain should be left in place 5 min. .+-.30 seconds as timing influences staining intensity. The Giemsa stain is then poured off and the plates are dipped three times in cold tap water in a beaker. The plates are then inverted, shaken vigorously, and air dried overnight (with plate lids off) on a rack on a laboratory bench. Cells per well are then counted manually or by automated and/or computerized means, to derive data regarding chemosensitivity of cells at various concentrations of exposure. One particularly useful computer operating environment for counting cells is the commercially available OPTIMATE compiler, which is designed to permit an optical counting function well suited to computerized cell counting procedures and subsequent calculations.

25 The above procedures do not change appreciably when cell growth promoters are assayed rather than cell arresting agents such as chemotherapeutic or radiotherapeutic agents. The present assay allows cell death or cell growth to be monitored with equal ease. In any case, optimization of use of the present system will involve the comparative testing of a variety of candidate active agents for selection of the best candidate for patient treatment based upon the in vitro results. One particularly advantageous embodiment of the above described invention comprises a two-stage assay for cytotoxicity followed by evaluation of longer-term inhibitory effect. Chemotherapeutic agents may thus be evaluated separately for both their direct chemotherapeutic effect as well as for their longer duration efficacy.

26 As discussed in brief, above, in parallel with growth of the prime or reference culture, a control culture can be grown. The control culture is a culture of normal cells taken from the same patient from whom the prime culture is collected. The control culture can derive from an epithelial cell sample or can be collected from the same organ as the prime culture so long as the control culture contains no malignant cells. More than one control culture can be maintained. For instance, cultures of both normal skin cells and normal cells of an organ from which the malignancy is derived can be maintained. The value of maintaining a control culture is many fold. Primarily, the control culture serves as a negative control (or positive control, depending upon the marker to be analyzed) in the various analyses to be carried out on the prime culture, the reference culture or subcultures thereof.

27 A second value of the control culture is an indicator of toxicity, the toxicity or undesirable effects of a given therapy upon normal cells. For instance, in

the segregated analysis of chemotherapeutic agents described above, concomitant analysis of the same agents on segregated sites of the control culture would yield an indication of cytotoxicity of the agent with regard to malignant cells versus the toxicity of the agent to control cells. A therapeutic index can be calculated based on the ratio of cytotoxicity to malignant cells to toxicity. Cytotoxicity and toxicity can be quantified as a percentage or fraction of cells killed by a given therapy, or as a percentage or fraction of cells surviving a given therapy. A therapeutic index is a ratio of these percentages or fractions and is reflective of the desirability of a given treatment in a patient. An optimal treatment would be maximally cytotoxic (or even cytostatic) to the malignant cells and minimally toxic to a patient's normal cells.

28 Other indices may be generated, depending upon the desired effect of a therapy. For instance, if a desired therapy is designed to up-regulate a malignancy-specific antigen to promote destruction of the malignancy by a patient's immune system, an index could be generated to discern a treatment which reflects maximal up-regulation of the antigen in the malignant cells and minimal or negative up-regulation in a patient's normal cells. A similar index can be calculated based upon down-regulation of a desired marker (i.e., an angiogenic factor) which can be assayed as either a secreted or a cellular marker and reflects maximal down-regulation of the marker with minimal toxicity or other undesirable effects on the control culture.

29 Often the diseased cells express a cellular marker that is indicative of a certain disease state or lack thereof. However, one aspect of the culture techniques of the present invention is that the cultured diseased cells do not necessarily have to be the cells expressing the factor to be assayed. One question that inevitably arises when considering whether a serum marker is indicative of a particular cancer cell is, which cells produce the marker, the cell or the tissue in which the cancer cells grow? See e.g. Singhal et al., p 610. By co-culturing the cancerous tissue within a multicellular particulate of its originating tissue, the cells (both the diseased cells or the surrounding cells) are better able to retain their production of characteristic markers.

30 Identification of one or more active agents or chemotherapeutic agents is peripheral to the present invention, which is intended for the efficacy screening of any or all of them as to a given patient. Literally any active agent may be screened according to the present invention; listing exemplary active agents is thus omitted here.

31 One important focus of the present invention thus includes the simplicity of the present system--cohesive multicellular particulates of the patient tissue to be tested are used to form cell monolayers; growth of those monolayers is monitored for accurate prediction of correlating growth of the same cells in vivo; and differing concentrations of a number of active agents may be tested for the purpose of determining not only the most appropriate agent but the most appropriate concentration of that agent for actual patient exposure (according to the calculated cell growth rates). It is also important to note, in the context of the invention, that the present system allows in vitro tests to be conducted in suspensions of tissue culture monolayers grown in nutrient medium under fast conditions (a matter of weeks), rather than with single cell progeny produced by dilution cloning over long periods of time. In some cases, the present invention is a two stage assay for both cytotoxicity and the longer-term growth inhibitory.

32 It is additionally possible to increase the value of the assay with the use of staining compositions and protocols designed to characterize the malignant cells thus grown. In other words, the tissue preparation and cell culturing technique itself offers a first assurance that the cells grown out of the tumor are really the malignant tumor cells and not fibroblasts or other nonmalignant cells of no diagnostic value. As a separate confirmation, the present staining compositions and protocols offer a second, independent assurance that the cells subject to diagnostic or prognostic assay are in fact malignant cells in culture. One important characterization has to do with the nature of the

malignant cells as epithelial, which is in turn an indicator of the carcinoma type of malignancy. Other characterizations of malignant cells are intended to fall within the scope of the present invention as well, although the characterization of the cells as epithelial or not is of primary importance.

33 The technique is practiced as follows. The same cell culturing and well distribution process is used as in the cytotoxicity assay described above, but rather than exposing the cells to chemotherapeutic or other agents, the cells are instead fixed and stained. With the stain or stain cocktail described below, the epithelial cells are identified by their intermediate filaments and/or specific membrane antigens by means of a monoclonal antibody immunoperoxidase technique. The fixative used can be any fixative which does not alter the cellular molecular markers of interest. The fixed, stained cells are then counted. If the specimen is positive for epithelial cells, the process is complete. If the specimen is negative for epithelial cells, an independent fixing and staining process is subsequently completed, with fresh cells from identical wells, using Vimentin as a stain to confirm the non-epithelial nature of the cells.

34 The importance of having a stain or stain cocktail (i.e., antibody cocktail), as well as an overall protocol, for identifying epithelial cells in biopsies of malignant tumors is as follows. In the basic cytotoxicity assay, the tissue culture technique is designed to grow out the cells of the tumor of origin and in fact consistently does so. Despite such reliable predictability, however, the fact that the cells of the tumor of origin did in fact grow out, and not fibroblasts or other cells, must be confirmed with independent proof before the cells can be used with complete assurance in the appropriate patient assay(s). The present technology provides a means to obtain this confirmation, which in turn furthers the interests of good laboratory and medical practice.

35 As a general consideration, the staining compounds or compositions of interest for use in the present technology are those which bind with cellular molecular markers unique either to epithelial or to non-epithelial cells. A further aspect of the invention therefore inheres in the following two aspects: the improvement of the cytotoxicity assay by adding the epithelial staining protocol with any known epithelial stain; and the further improvement wherein specially designed stain cocktails maximize the likelihood that the presence of any known intermediate filament or specific membrane antigen, characteristic of epithelial cells, will be identified if present.

36 Many carcinomas are positive for any one of the intermediate filaments or specific membrane antigens characteristic of epithelial cells; virtually all if not all carcinomas are positive for one of a number of such intermediate filaments or specific membrane antigens. For example, "epithelial membrane antigen" (EMA) glycoproteins are known in the art and can be bound with various antiepithelial membrane antigen antibodies including monoclonal antibodies. Cytokeratin is another important epithelial cell marker and binding reagents including monoclonal antibodies are available which are specific to cytokeratin. While antisera can be raised in vivo against markers such as EMA glycoproteins and cytokeratin, as a practical matter commercially available polyclonal or monoclonal antibodies are used in the following protocols, with monoclonal antibodies being preferred.

37 Binding of the epithelial marker is revealed with associated staining procedures and reactions which give a visual indication that the marker binding took place. Those skilled in the art already appreciate various techniques already available--in the general field of "immunocytochemistry"--to reveal antibody-antigen re-actions. One known way to accomplish this visualization when antibody binding reagents are used is with the "labeled streptavidin procedure". In this procedure, after the specimen is exposed to antibodies specific to the target antigen, a secondary "link" antibody is added. The secondary biotinylated "link" antibody consists of anti-mouse and anti-rabbit antibodies which bind universally to most primary monoclonal or polyclonal antibodies. The "link" will also connect to the tertiary reagent

(peroxidase-labeled streptavidin) through chemical bonding between the biotin on the secondary reagent and the streptavidin on the streptavidin/peroxidase conjugate. Staining is completed by incubating the specimen and primary, secondary and tertiary agents in the presence of a chromogen, so that the peroxidase and the chromogen form a visible precipitate. Alternatively, a fluorescein-based detection system can be used to visualize the primary antibody, or a third alternative known in the art as the digoxigenin-conjugated detection system may be used.

38 Of the various epithelial markers, three have received the most widespread attention in the literature: EMA glycoproteins, cytokeratin, and carcinoembryonic antigen. In the context of this invention, the first two are the most important because literally any epithelial cell will have at least either one EMA glycoprotein on the surface thereof or a cytokeratin intermediate filament present. Therefore, the present invention resides not only in binding and staining for an epithelial marker on the surfaces of the specimen cells, but in simultaneously assaying for either or both of EMA glycoprotein(s) and cytokeratin. The cocktails of the present invention therefore contain binding reagents for both EMA glycoproteins and cytokeratin and, importantly, are selected to include the most generally applicable binding reagents in combination so that the cocktail has the broadest binding scope possible. The cocktails identified in Examples 1 and 2, for example, represent a combination of two general binding reagents (containing a total of three monoclonal antibodies) for cytokeratin, admixed with a general binding reagent for EMA glycoprotein. The dual benefit of this admixture of general binding agents is that the incidence of false negatives for epithelial cells is minimized, and the visible staining reactions are generally stronger when the combined binding reagents are used in lieu of a single binding reagent.

39 Although the binding reagents and other reagents identified in the Examples are the preferred reagents for use in the context of the invention, the invention is intended to encompass epithelial-specific binding and staining reagents generally. These include, without limitation: Boehringer-Mannheim AE1 anti-cytokeratin antibody; Boehringer-Mannheim AE3 anti-cytokeratin antibody; Boehringer-Mannheim AE1/AE3 anti-cytokeratin antibody (AE1 and AE3 in admixture); Becton-Dickinson CAM 5.2 antibody, DAKO EMA antibody, Biomeda's Anti-Cytokeratin Cocktail CK22, Biomeda's Anti-Cytokeratin Cocktail CK23, Biomeda's Anti-Pan-Cytokeratin CK56, Biomeda's polyclonal goat or rabbit anti-cytokeratin antisera, ScyTek Laboratories' anti-EMA antigen antibody clone E29, and many others. Those skilled in the art and in possession of the guidance provided herein can readily determine alternative, equivalent binding and staining reagents and cocktails, to accomplish the disclosed result. These binding agents and cocktails may be used in combination with any known visualization system, such as the streptavidin, fluorescein- and digoxigenin-conjugated systems identified above.

40 As a control, Vimentin antibody is used as a binding alternative either in conjunction with binding and staining of the test cells, or subsequently thereto. In the context of this invention, Vimentin can be considered a binding reagent which is specific to non-epithelial cells of mesenchymal origin.

41 In a further aspect of the present invention, immunological markers may be monitored in applications requiring up- or down-regulation of such markers (i.e., Major histocompatibility complex molecules). This aspect of the present invention can be especially useful in transplantation applications where, for instance, through chemical or biological means rejection of transplanted cells is sought to be avoided by down-regulation of the various transplantation antigens present on the cells to be transplanted. The present invention would be especially useful in monitoring such immunoregulation.

42 Lastly, cell morphology can be assayed by culturing cells of, i.e., the prime culture or the reference culture, removing the cells from the surface upon which they grow, centrifuging cells into a loose pellet and growing the cell pellet over a defined time period. By growing cells in this manner, it is

possible to view the cohesive morphology of cells in a cluster resembling a tumor.

43 EXAMPLE 1

44 Radiation Therapy

45 Separate 50 mg samples from residual tissue from specimens of three human glioblastomas and one human ovarian carcinoma were minced in medium with sterile scissors to a particle size of roughly 1 mm.sup.3 and with a particle size distribution between about 0.25 and about 1.5 mm.sup.3. The medium was Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin. Each 50 mg sample was minced and was divided into four groups of particulates and each of 16 groups was charged to a separate labeled culture flask containing the above-described medium. Visual confirmation was made that the particulates were evenly distributed along the bottom of each flask and the flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the particulates grew into monolayers.

46 Enough cells were then removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets for each of the 16 flasks. Each cell pellet was then suspended in 5 ml of the above-described medium and was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10 times. A 36 ml droplet from the center of each tube was then pipetted into one well of a 96-well microtiter plate together with an equal amount of trypan blue, plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10.times. magnification--only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated and by means known in the art the optimum concentration of cells in the medium was determined.

47 Accommodating the above calculations, additional cell aliquots from the 16 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. Cells were plated into 60-well microtiter plates at a concentration of 100 cells per well.

48 Twenty-four (24) hours later, the cells were irradiated using a Siemens Stabilipan X-ray machine at 250 kVp, 15 mA with a dose rate of 75 rad/minute. For each radiation dose from 1 Gy to 6 Gy, cell number per well was monitored as a function of time through five days post-irradiation.

49 Cell number relative to controls was determined and survival curves were fit to the data. The rate of decrease in survival as a function of time was proportional to dose. A differential radiation response among the four cell lines was observed.

50 EXAMPLE 2

51 Immuno Therapy

52 Separate 50 mg samples from residual tissue from specimens of a human brain tumor, renal carcinoma, and breast carcinoma were minced in medium with sterile scissors to a particle size of roughly 1 mm.sup.3 and with a particle size distribution between about 0.25 and about 1.5 mm.sup.3. The medium was Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin. Each 50 mg sample was minced and was divided into four groups of particulates and each of 12 groups was charged to a separate labeled culture

flask containing the above-described medium. Visual confirmation was made that the particulates were evenly distributed along the bottom of each flask and the flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the particulates grew into monolayers.

53 Enough cells were then removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets for each of the twelve flasks. Each cell pellet was then suspended in 5 ml of the above-described medium and was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10 times. A 36 ml droplet from the center of each tube was then pipetted into one well a 96-well microtiter plate together with an equal amount of trypan blue, plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10.times. magnification--only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated and by means known in the art the optimum concentration of cells in the medium was determined.

54 Accommodating the above calculations, additional cell aliquots from the 12 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. Cells were plated into 60-well microtiter plates at a concentration of 100 cells per well.

55 Twenty-four (24) hours post-plating, Activated Natural Killer (ANK) cells were delivered into a row of six wells by means of a micropipette. In each microtiter plate three rows of six wells each served as controls. The effector (ANK cells):target cell (tumor cells) ratio varied from 2.5:1 to 20:1. The ANK cells were exposed to the target cells for four hours. Subsequently, the wells were washed with Hanks Balanced Salt Solution and the number of ANK cells remaining in the wells was observed with a phase contrast microscope. This process was repeated until no ANK cells remained in the wells (usually 3 washes). Following removal of the ANK cells, the tumor cells were incubated in the wells for another 24 hours.

56 Cell number relative to control was determined. For the three tumor types increasing the effector:target cell ratio from 2.5:1 to 20:1 resulted in an increase in the number of tumor cells killed by the ANK cells.

57 EXAMPLE 3

58 Gene Therapy/Antisense Oligonucleotides

59 A 50 mg sample from a residual human mesothelioma was minced in medium with sterile scissors to a particle size of roughly 1 mm.sup.3 and with a particle size distribution between about 0.25 and about 1.5 mm.sup.3. The medium was Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin. The 50 mg sample was minced and was divided into four groups of particulates and each of four groups was charged to a separate labeled culture flask containing the above-described medium. Visual confirmation was made that the particulates were evenly distributed along the bottom of each flask and the flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the particulates grew into monolayers.

60 Enough cells were then removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets for each of the four flasks. Each cell pellet was then suspended in 5 ml of the above-described medium and

was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10 times. A 36 ml droplet from the center of each tube was then pipetted into one well of a 96-well microtiter plate together with an equal amount of trypan blue, plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10.times. magnification--only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated and by means known in the art the optimum concentration of cells in the medium was determined.

61 Accommodating the above calculations, additional cell aliquots from the 4 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. Cells were plated into 60-well microtiter plates at a concentration of 100 cells per well.

62 Twenty-four (24) hours post-plating, antisense oligonucleotide for the urokinase-type plasminogen activator receptor (uPAR) was delivered to wells in the microtiter plate. Proteolysis of plasminogen to plasmin by urokinase-type plasminogen activator has been implicated in the processes of tumor cell proliferation and invasion. The concentrations of the uPAR antisense oligonucleotide were 1, 10 and 100 micromolar. uPAR sense and missense oligonucleotides at the concentrations of 1, 10 and 100 micromolar served as controls. The tumor cells were exposed to the oligonucleotides for 24 hours and then the agents were removed. The cells were allowed to incubate for another 72 hours so that inhibition of cell proliferation could be observed.

63 Cell number relative to control was then determined. Antisense oligbnucleotides to uPAR suppressed the proliferative activity of the tumor cells in a concentration dependent manner.

64 EXAMPLE 4

65 Combination Chemotherapy

66 Separate 50 mg samples from residual tissue from specimens from four human ovarian tumors were minced in medium with sterile scissors to a particle size of roughly 1 mm.sup.3 and with a particle size distribution between about 0.25 and about 1.5 mm.sup.3. The medium was Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin. Each 50 mg sample was minced and was divided into 4 groups of particulates and each of 16 groups was charged to a separate labeled culture flask containing the above-described medium. Visual confirmation was made that the particulates were evenly distributed along the bottom of each flask and the flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the particulates grew into monolayers.

67 Enough cells were then removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets for each of the 16 flasks. Each cell pellet was then suspended in 5 ml of the above-described medium and was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10 times. A 36 ml droplet from the center of each tube was then pipetted into one well of a 96-well microtiter plate together with an equal amount of trypan blue, plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10.times. magnification--only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated and by means known in the art the optimum concentration of cells in the medium was

determined.

68 Accommodating the above calculations, additional cell aliquots from the 16 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. Cells were plated into 60-well microtiter plates at a concentration of 100 cells per well.

69 Twenty-four (24) hours post-plating, the chemotherapeutic agent Taxol was applied to the wells in the microtiter plates. The first three treatment rows in the plates (Rows 2, 3, and 4) were designed to have escalating Taxol doses (1.0, 5.0, and 25 .mu.M) with a fixed carboplatin dose (200 .mu.M). The last three treatment rows in the plates (Rows 6, 7, and 9) were designed to have a fixed Taxol dose (5 .mu.M) with an escalating carboplatin dose (50, 200, and 1000 .mu.M). Rows 5 and 9 served as a control. The Taxol exposure time was two hours. Twenty-four hours later, the cells in the wells were exposed to carboplatin for two hours. The tumor cells in the wells were then incubated for another 48 hours.

70 Cell number relative to control was determined. For the cells from the four tumor specimens a dose response relationship was observed for both the escalating Taxol/fixed carboplatin and fixed Taxol/escalating carboplatin treatment schema.

71 EXAMPLE 5

72 Hormonal Therapy

73 Separate 50 mg samples from residual tissue from specimens from four human breast tumors were minced in medium with sterile scissors to a particle size of roughly 1 mm.sup.3 and with a particle size distribution between about 0.25 and about 1.5 mm . The medium was Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin. Each 50 mg sample was minced and was divided into four groups of particulates and each of 16 groups was charged to a separate labeled culture flask containing the above-described medium. Visual confirmation was made that the particulates were evenly distributed along the bottom of each flask and the flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the particulates grew into monolayers.

74 Enough cells were then removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets for each of the 16 flasks. Each cell pellet was then suspended in 5 ml of the above-described medium and was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10 times. A 36 ml droplet from the center of each tube was then pipetted into one well of a 96-well microtiter plate together with an equal amount of trypan blue, plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10.times. magnification--only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated and by means known in the art the optimum concentration of cells in the medium was determined.

75 Accommodating the above calculations, additional cell aliquots from the 16 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. Cells were plated into 60-well microtiter plates at a concentration of 100 cells per well.

76 Twenty-four (24) hours post-plating, the antiestrogenic compound Tamoxifen was delivered to wells in the microtiter plates. A stock solution of Tamoxifen was initially prepared by dissolving 1.5 mg of Tamoxifen powder in 1 ml of absolute ethanol and then adding 9 ml of growth medium. This stock solution was then used to make Tamoxifen solutions in the concentration range of 10 nM to 20 .mu.M. Six doses of Tamoxifen were used for cells from each of the four breast tumor specimens. An ethanol solution at a concentration equivalent to that at the highest Tamoxifen concentration served as a control. The tumor cells were exposed to Tamoxifen for 24 hours and then the agent was removed. The cells were allowed to incubate for another 72 hours so that inhibition of cell proliferation could be observed.

77 Cell number relative to control was then determined. There was no effect observed when the ethanol-only control wells were compared to the growth medium-only control wells. The cells of two of the four breast specimens tested showed an inhibition of cell proliferation by Tamoxifen exposure. These responses occurred in the mid to high Tamoxifen concentration ranges.

78 EXAMPLE 6

79 Differentiating Agent Therapy ("Biological Response Modification")

80 Separate 50 mg samples from residual tissue from specimens from four human breast tumors were minced in medium with sterile scissors to a particle size of roughly 1 mm.sup.3 and with a particle size distribution between about 0.25 and about 1.5 mm.sup.3. The medium was Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin. Each 50 mg sample was minced and was divided into four groups of particulates and each of 16 groups was charged to a separate labeled culture flask containing the above-described medium. Visual confirmation was made that the particulates were evenly distributed along the bottom of each flask and the flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the particulates grew into monolayers.

81 Enough cells were then removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets for each of the 16 flasks. Each cell pellet was then suspended in 5 ml of the above-described medium and was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10 times. A 36 ml droplet from the center of each tube was then pipetted into one well of a 96-well microtiter plate together with an equal amount of trypan blue, plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10.times. magnification--only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated and by means known in the art the optimum concentration of cells in the medium was determined.

82 Accommodating the above calculations, additional cell aliquots from the 16 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. Cells were plated into 60-well microtiter plates at a concentration of 100 cells per well.

83 Twenty-four (24) hours post-plating the differentiating agent retinoic acid was delivered to wells in the microtiter plates. A stock solution of retinoic acid was initially prepared by dissolving retinoic acid powder in 1 ml of dimethyl sulfoxide (DMSO) and then adding 9 ml of growth medium. This stock solution was then used to make retinoic acid solutions in the concentration range of 0.1 to 1.0 mM. Six doses of retinoic acid were used for cells from each of the four breast tumor specimens. A DMSO solution at a concentration equivalent to that

at the highest retinoic acid concentration served as a control. The tumor cells were exposed to retinoic acid for 24 hours and then the agent was removed. The cells were allowed to incubate for another 72 hours so that inhibition of cell proliferation could be observed.

84 Cell number relative to control was then determined. There was no effect observed when the DMSO-only control wells were compared to the growth medium-only control wells. The cells of three of the four breast specimens tested showed an inhibition of cell proliferation by retinoic acid exposure. These responses occurred in the mid to high retinoic acid concentration ranges.

85 EXAMPLE 7

86 Combined Modality Therapy Drug/Radiation

87 Separate 50 mg samples from residual tissue from specimens from two human brain tumors and two human ovarian tumors were minced in medium with sterile scissors to a particle size of roughly 1 mm.sup.3 and with a particle size distribution between about 0.25 and about 1.5 mm.sup.3. The medium was Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin. Each 50 mg sample was minced and was divided into four groups of particulates and each of 16 groups was charged to a separate labeled culture flask containing the above-described medium. Visual confirmation was made that the particulates were evenly distributed along the bottom of each flask and the flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the particulates grew into monolayers.

88 Enough cells were then removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets for each of the 16 flasks. Each cell pellet was then suspended in 5 ml of the above-described medium and was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10 times. A 36 ml droplet from the center of each tube was then pipetted into one well of a 96-well microtiter plate together with an equal amount of trypan blue, plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10.times. magnification--only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated and by means known in the art the optimum concentration of cells in the medium was determined.

89 Accommodating the above calculations, additional cell aliquots from the 16 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. Cells were plated into 60-well microtiter plates at a concentration of 100 cells per well.

90 Twenty-four (24) hours post-plating, cells in the microtiter plate wells were exposed to the chemotherapeutic agent Taxol. One set of plates was designed to have escalating Taxol doses with (0.5-25.0 .mu.M) with a fixed radiation dose (2 Gy). A second set of plates was designed to have a fixed Taxol dose (5 .mu.M) with an escalating radiation dose (1 Gy-6 Gy). The cells in the plates were irradiated using a Siemans Stabilipan X-ray machine operating at 250 kVp, 15 mA with a dose rate of 75 rad/minute.

91 For each of the two treatment schema, cell number per well was monitored as a function of time through 5 days post-treatment. Cell number relative to controls was determined and survival curves were fit. A differential response among the cells from the four tumor specimens was observed. Both additive and synergistic cell killing was noted.

92 EXAMPLE 8

93 Initiation of a Prime Culture

94 A tumor biopsy of approximately 100 mg of non-necrotic, non-contaminated tissue was harvested from the patient by surgical biopsy and transferred to the laboratory in a standard shipping container. Biopsy sample preparation proceeded as follows. Reagent grade ethanol was used to wipe down the surface of a Laminar flow hood. The tumor was then removed, under sterile conditions, from its shipping container, and cut into quarters with a sterile scalpel. Using sterile forceps, each undivided tissue quarter was then placed in 3 ml sterile growth medium (Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin) and was systematically minced by using two sterile scalpels in a scissor-like motion. The tumor particulates each measured about 1 mm.sup.3. After each tumor quarter was minced, the particles were plated in culture flasks using sterile pasteur pipettes (9 explants per T-25 or 20 particulates per T-75 flask). Each flask was then labeled with the patient's code, the date of explanation and any other distinguishing data. The explants were evenly distributed across the bottom surface of the flask, with initial inverted incubation in a 37.degree. C. incubator for 5-10 minutes, followed by addition of about 5-10 ml sterile growth medium and further incubation in the - normal, non-inverted position. Flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the explants grew out into a monolayer.

95 EXAMPLE 9

96 Unified Tracking System

97 a. Growth Rate

98 Following initiation of prime cell culture of a tumor specimen, the growth rate of the cells was determined until the chemosensitivity assay was performed. During this time period the growth was monitored by observing the percent of confluency of the cells in a flask. These data provide information valuable as a correlation to possible growth of the tumor in the patient as well as for the interpretation of the results of the chemosensitivity assay.

99 Three examples of growth rate data are shown in FIGS. 1A-1C. The percent of confluency of the cultured cells is plotted as a function of time after the initial seeding of the tissue specimen.

100 Slow Growth Rate (FIG. 1A): 25% confluent after 19 days Moderate Growth Rate (FIG. 1B): 60% confluent after 21 days Fast Growth Rate (FIG. 1C): 90% confluent after 11 days

101 b. Immunohistochemical Staining for Cell Characterization, etc.

102 Many tumor specimens will contain a mixture of cancer and normal cells. Although in many cases tumor cells will readily grow in tissue culture, while the normal cells will not, it is important to be able to distinguish the two cell types. Using immunoperoxidase techniques to stain cells for various intermediate filaments, the differences between normal (fibroblast-like) cells and cells from epithelial tumors were characterized. These techniques can also be used to identify other tumor cell characteristics which may have prognostic value.

103 An initial attempt at cultured cell characterization has been to use known epithelial tumor cell lines and a known fibroblast cell line. The epithelial tumor cell lines all have stained positively for a mixture ("cocktail") of

epithelial intermediate filament antibodies, (not every line, however, has stained positively for the three antibodies within the mixture [AE1/AE3; Cam 5.2; EMA]). Some of the epithelial tumor cells in culture also stained mildly positive for an antibody against an intermediate filament characteristic of fibroblasts (vimentin). When staining for fibroblast intermediate filament (vimentin) in cell culture, all fibroblast cells were positive. Some focal staining by epithelial tumor cells for vimentin was also present..

	epithelial cocktail	vimentin
epithelial tumor cells	++	+
fibroblasts	-	++

104 Testing of intermediate filaments with antibodies for epithelial cells and vimentin appears to be a method of distinguishing certain characteristics of tumor and normal cells.

105 c. Response to Chemotherapy

106 The tissue culture chemosensitivity assay has been refined to make it more sensitive for the detection of damage produced by a variety of chemotherapeutic agents. The initial alteration was to allow a 24-hour time period between plating of cells in microtiter wells and the exposure to drugs. This time interval permits cells to be in an active state of proliferation, where they are more sensitive to cell cycle active agents. The second change was to initiate a long-term assay (growth inhibition assay) over a period of about 72 hours. The short-term assay is conducted 24-72 hours after the therapeutic agent is added. The longer time between drug exposure and assay allows for the detection of cell damage which occurs over a protracted period and requires several cell division cycles before it becomes apparent. "CI" is a measure of the relative survival rates of a given cell culture. It is calculated by according to the formula: ##EQU2##

107 The data for a short-term assay and a long-term assay performed on two sets of patient cultured cells are presented in FIGS. 2A-2F through 5A-5F. The long-term assay (FIGS. 3A-3F and 5A-5F) may both accentuate a positive result obtained from the short-term assay (FIGS. 2A-2F and 4A-4F) and reveal an effect not observed during the short-term assay. The long-term assay is now incorporated into the tissue culture chemosensitivity on a routine basis.

108 d. Response to Radiation Therapy

109 The use of the microtiter well assay to analyze the direct effect of radiation therapy on tumor cells in culture has resulted in a rapid evaluation method for the determination of inherent cellular radiation response. As an example, two radiation dose-response curves generated from the microtiter well assay are presented in FIGS. 6 and 7. The cells from the tumor specimen in FIG. 6 are more resistant than those of the specimen in FIG. 7. The more resistant tumor has been previously irradiated.

110 The microtiter well assay is ideally suited for examination of the interaction of chemotherapeutic agents and radiation. Issues such as the differential sensitivity of drug/radiation combinations and the timing of drug/radiation combinations may be directly addressed with this system. An illustration of chemotherapeutic agent enhancement of radiation response is presented in FIGS. 8A-8C.

111 FIG. 8A: Radiation-only at 2 Gy and 4 Gy FIG. 8B: Taxol 8.5 ng/ml+2 Gy and 4 Gy

FIG. 8C: Taxol 42.5 ng/ml+2 Gy and 4 Gy

112 e. Response to Cellular Immunotherapy

113 Activated lymphocytes are being used as a treatment for some types of cancer. These Activated Natural Killers (ANK) cells have been shown to mediate highly efficient cell killing for some tumor types. The microtiter well assay can be utilized to make a rapid assessment of ANK-induced tumor target cell killing. An illustration of two such interactions is presented in FIGS. 9A and 9B.

114 In FIGS. 9A and 9B, the target cells were from a melanoma and a renal carcinoma, respectively. The target cells were exposed to the ANK cells for 4 hours and then the assay was performed. The effector:target cell ratio varied from 1:20 to 1:2.5. The data show increasing cell killing as a function of increasing effector:target ratio.

115 f. Use of Tissue Culture Medium for Determination of Factors with Possible Prognostic/Biological Significance

116 A number of substances secreted by tumor cells such as Tumor Associated Antigens and Plasminogen Activators and Inhibitors are believed to regulate a variety of processes involved in the progression of malignant disease. Many of these factors are produced by tumor cells growing in tissue culture and are secreted into the growth medium. The measurement of these factors in the medium from cell cultures of tumor specimens may prove to be of predictive value in the assessment of the biological behavior of individual cancers.

117 Preliminary work in this area has been on the detection of plasminogen activator inhibitor in the growth medium of glioblastoma cell lines. Plasminogen activator inhibitor expression has been shown to be increased in malignant brain tumors in patients. Medium from glioblastoma cell lines showed an increase in plasminogen activator inhibitor when compared to the medium alone.

118 Any or all of the steps of the unified assays and culturing techniques of the present invention may be automated. Indices can be automatically calculated by a computer which is programmed appropriately. Data can be input into the computer either manually or automatically, into a spreadsheet or database program, or the like. The spreadsheet or database program can be programed to reduce the data to the indices described above, or to any other relevant form, i.e., graphical or figurative representations of the data.

119 In one example, the cells to be assayed are grown on microtiter plates and assayed for their sensitivity to a chemotherapeutic agent according to the above-described protocols. The microtiter plates are read on an optical scanner and data from the scanner is automatically exported to a computer for calculation of a therapeutic index. Other types of scanners may be utilized depending upon the assay. For instance, a scanner for reading RIA data would be provided if the assay is an RIA assay.

120 Although the present invention has been described with respect to specific materials and methods above, the invention is only to be considered limited insofar as is set forth in the accompanying claims.

CLAIMS:

I claim:

1. A method of observing the behavior of cells derived from a sample of cancer cells, comprising:

(a) collecting a specimen of a patient's malignant or hyperproliferative cells;

(b) mechanically separating said specimen into cohesive multicellular particulates having a particle size between 0.25-1.5 mm.sup.3 ;

(c) growing a tissue culture monolayer from said multicellular particulates to form a prime culture which may be maintained;

(d) forming at least one subculture of said prime culture for further analysis; and

(e) monitoring said prime culture over a period of time for its characteristics to observe the behavior of the cells in said prime culture.

2. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 1, further comprising the step of maintaining the prime culture.

3. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 1, further comprising the steps of preparing a reference culture from the prime culture and treating the reference culture with one or more treatments as given to the patient.

4. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 3, further comprising the steps of preparing a subculture of one of the prime culture and the reference culture.

5. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 4, further comprising the step of assaying for a malignancy-specific marker in one of the prime culture, the reference culture, the subculture and tissue culture medium used to grow one of the prime culture, the reference culture or the subculture.

6. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 5, wherein the marker indicates one of aggressiveness and invasiveness of the cells in said prime culture.

7. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 6, wherein the marker is vascular endothelial growth factor.

8. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 5, wherein the marker is indicative of complications associated with the cells in said prime culture.

9. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 8, wherein the marker is indicative of a thrombogenic potential.

10. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 5, wherein the marker is identified by one of cytochemistry or immunohistochemistry.

11. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 10, wherein the marker is selected from the group consisting of an estrogen receptor, a progesterone receptor, an oncogene, a product of an oncogene, a marker for multi-drug resistance and a marker for phenotypic characterization.

12. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 5, wherein one or more of the steps are at least partially automated.

13. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 5, wherein the marker is characterized by a molecular

biological technique.

14. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 13, wherein the molecular biological technique characterizes one of tumor cell heterogeneity or specific mutations of cancer-related genes.

15. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 2, further comprising the steps of:

f. inoculating cells from one of the prime culture, the reference culture or a subculture of the prime culture or of the reference culture into a plurality of segregated sites; and

g. treating the plurality of sites with at least one treating means, followed by assessment of sensitivity of cells in the site to the treating means.

16. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 15, wherein one or more of the steps are at least partially automated.

17. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 15, further comprising the step of phenotypically or genotypically analyzing the cells in one or more sites for drug resistance.

18. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 15, further comprising the steps of:

h. collecting a specimen of a patient's non-malignant cells;

i. separating the non-malignant cells into cohesive multicellular particulates;

j. growing a tissue culture monolayer from the multicellular particulates of non-malignant cells to form a control culture;

k. inoculating the control culture in a plurality of non-segregated sites;

l. treating the plurality of segregated sites of the control culture with the same treating means as the segregated sites of the prime culture or a subculture thereof, followed by assessment of the sensitivity of the segregated cells of the control culture in the treating means; and

m. comparing the sensitivity of the segregated cells of the prime culture or a subculture thereof with the sensitivity of the segregated cells of the control culture to the treating means.

19. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 18, wherein the assessment of steps g and l are calculations of the percentage or fraction of cells sensitive to the treatment and further comprising the step of:

n. creating a therapeutic index of a ratio of one of the percentage of or the fraction of sensitive cells or insensitive cells in the segregated cells of the control culture to one of the percentage of or the fraction of sensitive cells or insensitive cells in the segregated cells of the prime culture or subculture thereof.

20. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 19, further comprising the step of programming a computer to automatically perform calculations to create said therapeutic index.

21. A method of observing the behavior of cells derived from a sample of cancer

cells as claimed in claim 20, wherein the segregated sites are in a readable plate having a plurality of culture wells and a scanner is used to automatically scan the segregated sites to determine the percentage or fraction of cells sensitive to the treatment and an interface is provided between the scanner and the computer allowing automated input of scanner data into the computer for calculation of the therapeutic index.

22. A method of observing the behavior of cells derived from a sample of cancer cells as claimed in claim 19, wherein the non-malignant cells are epithelial cells.

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ABSTRACT:

A comprehensive and integrated system for monitoring (identifying, tracking and analyzing) an individual patient's malignancy through the duration of a malignancy as to a specific patient is provided. The method of the present invention allows for initial identification of a malignancy, identification of malignancy-specific cellular or secretal markers, identification of cellular or secreted markers indicative of complications, study of the invasiveness and aggressiveness of the malignancy, study of the growth rate of the malignancy, study of the effect of therapies on the malignancy as compared to control cells of the same patient (chemosensitivity versus toxicity) and the identification of a therapeutic index (i.e., the ratio of chemosensitivity:toxicity), study of tumor morphology and study of histological, cytochemical and immunocytochemical markers.

RELATED APPLICATION

[0001] This is a Continuation-In-Part of U.S. application Ser. No. 08/679,056, filed Jul. 12, 1996, now U.S. Pat. No. 5,728,541, granted Mar. 17, 1998; U.S. application Ser. No. 09/095,993, filed Jun. 11, 1998; and U.S. application Ser. No. 09/039,957, filed Mar. 16, 1998.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] A system is provided for in vitro tracking of cancerous tissue over the course of the malignancy. The system provides a method for identifying the malignancy and for determining a patient's prognosis. Further, the system provides for assessing a malignancy's invasiveness, aggressiveness, growth rate, production of extracellular markers, possible side effects and for determining the efficacy on the malignancy of a given therapeutic regimen. The system also allows for generation of a therapeutic index, which serves as an indicator of a given therapy's effectiveness against the malignancy as compared to its undesirable side effects, such as lethality to a patient's normal cells.

Introduction

[0004] Tracking a malignancy in a patient according to prior art methods is an inaccurate process which involves identification of the malignancy through techniques including biopsy and subsequent histological, biochemical, and immunochemical techniques and regularly monitoring the malignancy's progression by invasive (i.e., biopsy) or noninvasive (i.e., x-ray, nuclear imaging, Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET)) methods. These methods are often expensive, inconvenient, painful and usually involve hospital visits and safety risks. It is, therefore, desirable to reduce a patient's exposure to such methods. Furthermore, identification of a malignancy as a known variety of malignancy is often helpful in determining a suitable therapeutic approach and expected prognosis. However, even individually identifiable malignancies differ from patient-to-patient in their growth characteristics and in their responsiveness to treatment.

[0005] Determination of the growth rate, invasiveness and aggressiveness of a given malignancy is critical to prognosis and to the choice of therapies. A patient with a poor prognosis might be given a therapeutic regimen which might be more effective than another regimen but more risky to the patient. A patient with a better prognosis might be given a therapeutic regimen which is less aggressive and less risky to the patient, but which might not be as effective as often as a more dangerous therapy. Similarly, if a malignancy produces factors or creates conditions which cause a dangerous side effect, such as a thrombogenesis, the patient can be treated, preferably prophylactically, for the condition.

[0006] Current methodologies for determining growth rate, invasiveness, aggressiveness or which track the progression of a malignancy include biopsy and short-term culture, which can include drawing of blood or other bodily fluids, or semi- or non-invasive techniques such as x-ray and nuclear imaging. At any given time, a patient could be subject to multiple procedures, depending upon when the information is needed by the physician. Each procedure requires the presence of the patient and usually creates risk or pain. These procedures also can increase the stress level of the patient, which often is an exacerbating factor in cancer and associated prognoses. It is therefore, desirable to reduce the frequency of such procedures.

[0007] Identification of an effective therapeutic regimen is critically important to a patient. Often, once the malignancy is identified, a therapy is chosen based upon prior research on that type of malignancy and is not tailored to the sensitivities of the malignancy of a given patient. Often secondary therapies are needed because a first choice was ineffective. Valuable treatment time can be lost and a patient's life can be threatened.

[0008] All active agents including chemotherapeutic active agents are subjected to rigorous testing as to efficacy and safety prior to approval for medical use in the United States. Methods of assessing efficacy have included elaborate investigations of large populations in double blind studies as to a given treatment method and/or active agent, with concomitant statistical interpretation of the resulting data, but these conclusions are inevitably generalized as to patient populations taken as a whole. In many pharmaceutical disciplines and particularly in the area of chemotherapy, however, the results of individual patient therapy may not comport with generalized data--to the detriment of the individual patient. The need has been long recognized for a method of assessing the therapeutic potential of active agents, including but not limited to chemotherapeutic agents, for their efficacy as to a given individual patient, prior to the treatment of that patient. This need also applies to assessing the therapeutic potential as to radiation therapies, combined radiation/drug therapies and cellular immunotherapies.

[0009] Prior art assays already exist which expose malignant tissue of various types to a plurality of active agents, for the purpose of assessing the best choice for therapeutic administration. For example, in Kruczynski, A., et al., "Evidence of a direct relationship between the increase in the in vitro passage number of human non-small-cell-lung cancer primocultures and their chemosensitivity," Anticancer Research, vol. 13, no. 2, pp. 507-513 (1993), chemosensitivity of non-small-cell-lung cancers was investigated in in vivo grafts, in in vitro primocultures and in commercially available long-term cancer cell lines. The increase in chemosensitivity was documented and correlated with morphological changes in the cells in question. Sometimes animal model malignant cells and/or established cell cultures are tested with prospective therapy agents, see for example Arnold, J.T., "Evaluation of chemopreventive agents in different mechanistic classes using a rat tracheal epithelial cell culture transformation assay," Cancer Res., vol. 55, no. 3, pp. 537-543 (1995).

[0010] In vitro prior art techniques present the further shortcoming that assayed cells do not necessarily express the cellular markers they would express in vivo. This is regrettable because the determination of expression of certain secreted or cellular markers, secreted factors or tumor antigens or lack thereof can be useful for both identification and therapeutic purposes. For instance, members of the fibrinolytic system such as urokinase-type plasminogen activator (u-PA) and plasminogen activator inhibitors type 1 (PAI-1) are up-regulated in malignant brain tumors. See, e.g., Jasti S. Rao, et al., "The Fibrinolytic System in Human Brain Tumors: Association with Pathophysiological Conditions of Malignant Brain Tumors," Advances in Neuro-Oncology II, Kornblith PL, Walker MD (eds) Futura (1997). Other secreted factors such as .alpha.-fetoprotein, carcinoembryonic antigen and transforming growth factors .alpha. and .beta. have been found to be indicative of various cancers and/or cancer progression (see also, Singhal et al., "Elevated Plasma Osteopontin in Metastatic Breast Cancer Associated with Increased Tumor Burden and Decreased Survival," Clinical Cancer Research, vol. 3, 605-611, (April 1997); Kohno et al., "Comparative Studies of CAM 123-6 and Carcinoembryonic Antigen for the Serological Detection of Pulmonary Adenocarcinoma," Cancer Detection and Prevention, 21(2): 124-128 (1997)). These examples are but a few of the many factors that may be used to identify diseased cells.

[0011] Cellular markers also include metastatic markers, indicative of metastatic potential, i.e., invasiveness and aggressiveness, which is relevant to the progression of a given malignancy and to a patient's prognosis. First, markers indicating the invasiveness of a given malignancy indicate the ability of the malignancy to infiltrate and to destroy adjacent tissue. As an example, for epithelial malignancies, invasiveness markers are indicative of the ability of the malignancy to infiltrate beneath the epithelial basement membrane. Invasiveness markers can include the presence of proteolytic enzymes or angiogenic factors. A second category of metastatic marker indicates growth conditions of the malignancy. For instance, a malignancy could require for instance a prostate-specific factor for growth. Invasiveness and aggressiveness factors are often present in serum or in tissue culture media.

[0012] Relevant to a patient's prognosis and, incidentally, to the identification of a malignancy is the presence of markers, cellular or secreted, which lead to complications beyond those involved with uncontrolled growth and invasion by a malignancy. For instance, secretion by the malignancy of thrombogenic substances by the malignancy can result in blood clotting, resulting in thrombophlebitis or other thrombotic events such as pulmonary thrombosis. Identification of a thrombotic potential indicates treatment (preferably prophylactically) with thrombolytic substances.

[0013] When a specific patient's cells are used in in vitro assays in typical prior art processes the cells are harvested (biopsied) and trypsinized (connective tissue digested with the enzyme trypsin) to yield a cell suspension purportedly suitable for conversion to the desired tissue culture form. The in vitro tissue culture cell collections which result from these techniques are generally plagued by their inability accurately to imitate the chemosensitivity or therapeutic sensitivity of the original tumor or other cell biopsy. These collections often do not express cellular markers in the same manner that they would in vivo. A need thus remains for a technique of tissue culture preparation which provides cell cultures, allowing identification of a malignancy, accurate tracking of the malignancy's progress in a patient and therapy screening, in which, after simple preparation, the cell cultures

react in a manner equivalent to their in vivo reactivity. The culture method would enable drug or chemotherapeutic agent, radiation therapy and/or cellular immunotherapy screening as to a particular patient for whom such screening is indicated.

[0014] A need also remains for a technique of tissue culture preparation which provides cell cultures for screening for expressed markers or factors where the cultured cells express the markers or factors in a manner indicative of their in vivo expression of the same. A further need also remains for a tissue culture preparation which allows for morphological study of the cells. Lastly, a need remains for a tissue culture system in which progression of an individual malignancy can be studied as indicative of the in vivo progression of the malignancy.

SUMMARY OF THE INVENTION

[0015] A comprehensive and integrated unified system for monitoring (i.e., identifying, tracking and analyzing) an individual patient's malignancy through the duration of a malignancy as to a specific patient is provided. The method of the present invention allows for initial identification of a malignancy, identification of malignancy-specific cellular or secreted markers, identification of cellular or secreted markers indicative of complications, study of the invasiveness and aggressiveness of the malignancy, study of the growth rate of the malignancy, study of the effect of therapies on the malignancy as compared to control cells of the same patient (chemosensitivity versus toxicity) and the identification of a therapeutic index (i.e., the ratio of chemosensitivity:toxicity), study of tumor morphology and study of histological and cytochemical markers.

[0016] The method of the present invention includes the steps of collecting a tissue sample or specimen of a patient's cells and separating the specimen into cohesive multicellular particulates (explants) of the tissue sample, rather than enzymatically digested cell suspensions or preparations. The cells are then grown as a tissue culture monolayer from the multicellular particulates to form a prime culture. A specimen can be taken from a patient at any relevant site, including but not limited to tissue, ascites or effusion fluid. Samples may also be taken from body fluid or exudates, as is appropriate. A tissue culture monolayer, designated as the prime culture, can be grown in any method known in the art for growing such a monolayer, for instance in tissue culture plates or flasks. If the malignant cells originate from solid tissue, however, the tissue must be subdivided into small pieces from which a tissue culture monolayer is then grown out.

[0017] Once a prime culture is established from a patient's malignancy, the prime culture can be maintained without any treatments beside normal feedings and passage techniques, as indicative of the growth of the malignancy absent treatment. However, subcultures of the prime culture are prepared so that the prime culture is preferably left untreated, and the cells of the prime culture are not affected by any testing. However, either the prime culture or a subculture thereof can be propagated as a reference culture. The reference culture is a culture which is treated with therapies reflective of a patient's actual treatments. For instance, if a patient is treated with a chemotherapeutic agent, the reference culture is treated with the same agent in the same concentration. The reference culture can be monitored genotypically or phenotypically to reflect actual progress of the malignancy in the patient. Treatment of the reference culture need not be limited to anticancer therapies, but can reflect all of a patient's treatments. For instance, thrombolytic or anti-thrombogenic treatments can be applied to the reference culture to reflect a patient's treatment. Subcultures of either the prime culture or the reference culture can be used for further analysis. Preferably, since the reference culture is indicative of the current state of a malignancy at a given time, subcultures of the reference culture are analyzed further. At various points in the passage of the control culture and the reference culture, aliquots of cells from those cultures can be stored cryogenically or otherwise.

[0018] The tissue sample technique of the present invention is also useful in assaying expression and/or secretion of various markers, factors or antigens present on or produced by the cultured cells. These assays can be used for diagnostic purposes for monitoring the applicability of certain candidate therapeutic or chemotherapeutic agents or for monitoring the progress of treatment of the cancer with those agents.

[0019] A method for identifying and monitoring progress of a malignancy in an

individual patient is provided including the steps of inoculating cells from either the prime culture, the reference culture or a subculture of the prime culture or of the reference culture into a plurality of segregated sites; treating the plurality of sites with at least one treating means or therapy, followed by assessment of sensitivity of cells in the site to the treating means; collecting a specimen of a patient's non-malignant cells; separating the non-malignant cells into cohesive multicellular particles; growing a tissue culture monolayer from the multicellular particulates of non-malignant cells to form a control culture; inoculating the control culture in a plurality of non-segregated sites; treating the plurality of segregated sites of the control culture with the same treating means as the segregated sites of the prime culture or a subculture thereof, followed by assessment of the sensitivity of the segregated cells of the control culture to the treating means; and comparing the sensitivity of the segregated cells of the prime culture or a subculture thereof with the sensitivity of the segregated cells of the control culture to the treating means. The assessments described above are calculations of the percentage or fraction of cells sensitive, or insensitive, to the treatment and the method further includes the step of creating a therapeutic index of a ratio of one of the percentage of or the fraction of sensitive cells or insensitive cells in the segregated cells of the control culture to one of the percentage of or the fraction of sensitive cells or insensitive cells in the segregated cells of the prime culture or subculture thereof.

[0020] Lastly, a method for treating a patient having a malignancy is provided, including the steps of: analyzing a patient's cells prepared according to the above-described methods for malignancy-associated markers; determining a therapeutic regimen according to the results of the analysis; and treating a patient according to the regimen. The method can further include the step of treating one of either cells cultured as a subculture of the prime culture or cells of the prime culture according to the regimen as representative of the patient's malignancy. Lastly, the method further includes determining a therapeutic index for each treating means as described above.

[0021] When applicable, cultures can be grown in a readable (scannable) plate and to determine percent confluence of the cells or any other parameter which can be determined in such a manner. The scanner can be operably linked with a computer or CPU to automatically input data into the computer or CPU. The computer or CPU can be programmed to automatically calculate a therapeutic index (or other relevant indices) based upon the data provided by the scanner. Alternatively, the data can be entered manually into the programmed computer or CPU to calculate the index.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] FIGS. 1A, 1B and 1C are graphs of the growth rates of three independent cell cultures.

[0023] FIGS. 2A-2F through 5A-5F are graphs depicting the results of short-term and long-term chemotherapy assays. FIGS. 2A-2F and 3A-3F show short-term and long-term assays for a first patient. FIGS. 4A-4F and 5A-5F show short-term and long-term assays for a second patient.

[0024] FIGS. 6 and 7 show two radiation dose versus surviving fraction curves for two glioblastoma cell lines. Cells were irradiated in microtiter plates and assayed four days post-irradiation.

[0025] FIGS. 8A-8C are graphs of survival rates of cell cultures treated with radiation (FIG. 8A) or with radiation and Taxol (FIGS. 8B and 8C).

[0026] FIGS. 9A and 9B are graphs showing data from a series of experiments where target cells from two tumor types were exposed to Activated Natural Killer (ANK) cells.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0027] The present invention is an improved and unified system for monitoring the progression of an individual malignancy and for identifying cellular and secreted markers, markers indicative of certain side effects of the malignancy and for screening multiple candidate therapeutic or chemotherapeutic agents for efficacy and long term effect as to a specific patient. In the method, a tissue sample from the patient is harvested, cultured and separately exposed to a plurality of treatments

and/or therapeutic agents for the purpose of objectively identifying the best treatment for the cultured cells obtained from the patient. The culture techniques of the present invention also result in a monolayer of cells that express cellular markers, secreted factors and tumor antigens in a manner representative of their expression in vivo. Specific method innovations such as tissue sample preparation techniques render this method practically as well as theoretically useful. One particularly important tissue sample preparation technique is the initial preparation of cohesive multicellular particulates of the tissue sample, rather than enzymatically dissociated cell suspensions or preparations, for initial tissue culture monolayer preparation. With respect to the culturing of malignant cells, for example, it is believed (without any intention of being bound by the theory) that by maintaining the malignant cells within a multicellular particulate of the originating tissue, growth of the malignant cells themselves is facilitated versus the overgrowth of fibroblasts or other cells which tends to occur when suspended tumor cells are grown in culture. Practical monolayers of cells may thus be formed to enable meaningful screening of a plurality of treatments and/or agents as well as meaningful identification of cellular markers. In the drug assays, growth of cells is monitored to ascertain the time to initiate the assay and to determine the growth rate of the cultured cells; sequence and timing of drug addition is also monitored and optimized. By subjecting uniform samples of cells to a wide variety of active agents (and concentrations thereof), the most efficacious agent can be determined. For assays concerning cancer treatment, a two-stage evaluation is contemplated in which both acute cytotoxic and longer term inhibitory effects of a given anti-cancer agent are investigated.

[0028] With regard to the identification of expressed cellular markers, secreted factors or tumor antigens, with the initial culturing of the multicellular particulates it is believed (without any intention of being bound by the theory) that because the cells are grown under conditions closer to those found in vivo, the cells express their cellular markers, secreted factors and tumor antigens in a manner more closely resembling their expression in vivo. By assaying the culture media obtained from growing a monolayer according to the inventive method or by histochemically and/or immunohistochemically assaying the cells grown under such conditions, a more accurate profile of the cellular markers or factors is obtained.

[0029] Thus, a comprehensive and integrated system for identifying, tracking and analyzing an individual patient's malignancy through the duration of the malignancy and thereafter is provided. The method of the present invention allows for initial identification of a malignancy, identification of malignancy-specific cellular or secreted markers, identification of cellular or secreted markers indicative of complications, study of the invasiveness and aggressiveness of the malignancy, study of the growth rate of the malignancy, study of the effect of therapies on the malignancy as compared to control cells of the same patient (chemosensitivity and/or radiosensitivity versus toxicity) and the identification of a therapeutic index (i.e., the ratio of chemosensitivity:toxicity), study of tumor morphology and study of histological, cytochemical and immunocytochemical markers.

[0030] The method of the present invention includes the steps of collecting a tissue sample or specimen of a patient's cells and separating the specimen into cohesive multicellular particulates (explants) of the tissue sample, rather than enzymatically digested cell suspensions or preparations. The cells are then grown as a tissue culture monolayer from the multicellular particulates to form a prime culture. A specimen can be taken from a patient at any relevant site, including but not limited to tissue, ascites or effusion fluid. Samples may also be taken from body fluid or exudates, as is appropriate. A tissue culture monolayer can be grown in any method known in the art for growing such a monolayer, for instance in tissue culture plates or flasks.

[0031] Once a prime culture is established from a patient's malignancy, the prime culture can be maintained without any treatments beside normal feedings and passage techniques, as indicative of the growth of the malignancy absent treatment with a therapeutic regimen. Subcultures of the prime culture are prepared so that the cells of the prime culture are not affected by any subsequent testing or treatments. Although prime culture is preferably left untreated, either the prime culture or a subculture thereof can be propagated as a reference culture. The reference culture is a culture which is treated with therapies reflective of a patient's actual treatment regimen. For instance, if a patient is treated with a chemotherapeutic agent, the reference culture is treated with the same agent in the same concentration. The reference culture can be monitored genotypically or

phenotypically to reflect actual progress of the malignancy in the patient. Treatment of the reference culture need not be limited to anticancer therapies, but can reflect all of a patient's treatments. For instance, thrombolytic or anti-thrombogenic treatments, can be applied to the reference culture to reflect a patient's treatment. Subcultures of either the prime culture or the reference culture can be used for further analysis. Preferably, since the reference culture is indicative of the current state in a patient of a malignancy, subcultures of the reference culture are analyzed. At various points in the passage of the control culture and the reference culture, aliquots of cells from those cultures can be stored cryogenically, or otherwise.

[0032] An important further aspect of the present invention is to provide a system for screening specific tissue samples from individual patients for expressed cellular markers, secreted factors or antigens, including tumor antigens, characteristic of the tissue sample. A tissue sample from a patient is harvested and grown in a monolayer culture as described above. Culture medium in which the cultures or subcultures thereof are assayed for the presence or absence of certain factors, such as secreted tumor antigens such as PAI-1, u-PA, cancer associated serum antigen (CASA) or carcinoembryonic antigen (CEA). These factors may be detected through use of standard assays such as radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA), although other assays known to those skilled in the art may be used to detect and/or to quantify the soluble factors. The cell cultures grown in this manner may also be assayed histochemically and or immunohistochemically for identification or quantification of cellular or membrane-bound markers. Examples of such markers include, without limitation, CEA, tissue polypeptide specific antigen, (TPS) and mucin antigens, such as CA 15-3, CA 549, CA 27.29 and MCA. By screening tissue samples in this manner, for production of such factors, markers or antigens, the cultured cells may be further identified, aiding the physician in treatment strategies and as a prognosis indicator. Furthermore, by combining the use of the culture technique with assaying for such markers, factors and antigens, a treatment strategy for a disease state may be optimized and treatment progression may be monitored.

[0033] One important aspect of analyzing tissue culture medium is that complications of a malignancy can be predicted. For instance, one common complication is thrombogenesis. A propensity towards blood clot formation can be detected in tissue culture medium by identifying thrombogenic or procoagulant factors such as, without limitation, the Lewis Y antigen (Ley), HLA-DR and other tumor procoagulants, such as cancer procoagulant (CP) and tissue factor (TF). By identifying production of thrombogenic factors, a physician can prescribe drug and/or exercise regimens, as appropriate, to prevent life and/or limb-threatening clotting.

[0034] Cells and/or tissue culture media from any of the prime culture, the reference culture or subcultures thereof can be analyzed for tumor aggressiveness and invasiveness markers. Presence of these markers or absence thereof is highly relevant to a patient's prognosis. Furthermore, the effect of a given therapy on any of these markers can be analyzed. For instance, a tumor may produce angiogenic factors, such as, without limitation, vascular endothelial growth factor (VEGF), which would lead a doctor to give a patient a less favorable prognosis. Other markers can include, without limitation, factors which allow cancer cells to affix to organs other than those from which the cancer cells derive, for instance, beta 3 integrin, which participates in the ability of melanoma cells to adhere to blood vessel walls. However, the effectiveness of therapies can be assessed if the presence of the angiogenic marker is analyzed in segregated sites according to the method of the present invention. A physician can suppress a malignancy by preventing expression of factors or markers which cause the malignancy's aggressiveness or invasiveness.

[0035] An important application of the present invention is the screening of chemotherapeutic agents and other anti-neoplastic therapies in tissue culture preparations of malignant cells from the patients from whom malignant samples are biopsied. Related anti-cancer therapies which also can be screened using the inventive system include radiation therapy and agents which enhance the cytotoxicity of radiation, as well as immunotherapeutic anti-cancer agents. Screening processes for treatments or therapeutic agents for nonmalignant syndromes are also embraced within this invention and include without limitation agents which combat hyper-proliferative syndromes, such as psoriasis, or wound healing agents. Nor is the present efficacy assay limited only to the screening of active agents which speed up (healing) or slow down (anti-cancer, anti-hyper-proliferative) cell growth

because agents intended to enhance or to subdue intracellular biochemical functions may be tested in the present tissue culture system also. For example, the formation or blocking of enzymes, neurotransmitters and other biochemicals may be screened with the present assay methods prior to treatment of the patient.

[0036] When a patient is to be treated for the presence of tumor, in the preferred embodiment of the present invention a tumor biopsy of >100 mg of non-necrotic, non-contaminated tissue is harvested from the patient by any suitable biopsy or surgical procedure known in the art. Biopsy sample preparation generally proceeds as follows under a Laminar Flow Hood which should be turned on at least 20 minutes before use. Reagent grade ethanol is used to wipe down the surface of the hood prior to beginning the sample preparation. The tumor is then removed, under sterile conditions, from the shipping container and is minced with sterile scissors. If the specimen arrives already minced, the individual tumor pieces should be divided into four groups. Using sterile forceps, each undivided tissue quarter is then placed in 3 ml sterile growth medium (Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin) and systematically minced by using two sterile scalpels in a scissor-like motion, or mechanically equivalent manual or automated opposing incisor blades. This cross-cutting motion is important because the technique creates smooth cut edges on the resulting tumor multicellular particulates. Preferably but not necessarily, the tumor particulates each measure 1 mm.sup.3. After each tumor quarter has been minced, the particles are plated in culture flasks using sterile pasteur pipettes (9 explants per T-25 or 20 particulates per T-75 flask). Each flask is then labeled with the patient's code, the date of explanation and any other distinguishing data. The explants should be evenly distributed across the bottom surface of the flask, with initial inverted incubation in a 37.degree. C. incubator for 5-10 minutes, followed by addition of about 5-10 ml sterile growth medium and further incubation in the normal, non-inverted position. Flasks are placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks should be checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the explants will foster growth of cells into a monolayer. With respect to the culturing of malignant cells, it is believed (without any intention of being bound by the theory) that by maintaining the malignant cells within a multicellular particulate of the originating tissue, growth of the malignant cells themselves is facilitated versus the overgrowth of fibroblasts (or other unwanted normal cells) which tends to occur when suspended tumor cells are grown in culture.

[0037] The use of the above procedure to form a cell monolayer culture maximizes the growth of malignant cells from the tissue sample, and thus optimizes ensuing tissue culture assay of chemotherapeutic action of various agents to be tested. Enhanced growth of actual malignant cells is only one aspect of the present invention; however, another important feature is the growth rate monitoring system used to oversee growth of the monolayer once formed. Once a primary culture and its derived secondary monolayer tissue culture has been initiated, the growth of the cells is monitored to ascertain the time to initiate the chemotherapy assay and to determine the growth rate of the cultured cells.

[0038] Monitoring of the growth of cells is conducted by counting the cells in the monolayer on a periodic basis, without killing or staining the cells and without removing any cells from the culture flask. The counting may be done visually or by automated methods, either with or without the use of estimating techniques known in the art (counting in a representative area of a grid multiplied by number of grid areas, for example). Data from periodic counting is then used to determine growth rates which may or may not be considered parallel to growth rates of the same cells in vivo in the patient. If growth rate cycles can be documented, for example, then dosing of certain active agents can be customized for the patient. The same growth rate can be used to evaluate radiation treatment periodicity, as well. It should be noted that with the growth rate determinations conducted while the monolayers grow in their flasks, the present method requires no hemocytometry, flow cytometry or use of microscope slides and staining, with all their concomitant labor and cost.

[0039] Protocols for monolayer growth rate generally use a phase-contrast inverted microscope to examine culture flasks incubated in a 37.degree. C. (5% CO.sub.2) incubator. When the flask is placed under the phase-contrast inverted microscope, ten fields (areas on a grid inherent to the flask) are examined using the 10.times. objective, with the proviso that the ten fields should be non-contiguous, or significantly removed from one another, so that the ten fields are a representative sampling of the whole flask. Percentage cell occupancy for each field examined is

noted, and averaging of these percentages then provides an estimate of overall percent confluency in the cell culture. When patient samples have been divided between two or among three or more flasks, an average cell count for the total patient sample should be calculated. The calculated average percent confluency should be entered into a process log to enable compilation of data--and plotting of growth curves--over time. Monolayer cultures may be photographed to document cell morphology and culture growth patterns. The applicable formula is: 1 Percent confluency = estimate of the area occupied by cells total area in an observed field.

[0040] As an example, therefore, if the estimate of area occupied by the cells is 30% and the total area of the field is 100%, percent confluency is 30/100, or 30.

[0041] Adaptation of the above protocol for non-tumor cells is straightforward and generally constitutes an equivalent procedure.

[0042] Active agent and/or radiation therapy screening using the cultured cells proceeds with subcultures of the prime culture or, preferably, of the reference culture. The screening can be carried out in an incubation flask, but generally proceeds using plates such as microtiter plates. In a chemotherapy/radiotherapy assay, it is desirable to grow a control culture of a patient's cells in a culture parallel to the reference or prime culture. The control culture can be grown from skin cells, as an easy source of non-malignant cells, from the same organ from which the malignant cells are derived, or from other sources, so long as the cells are typical of non-malignant cells of the patient.

[0043] The performance of the chemosensitivity/radiosensitivity assay used for screening purposes depends on the ability to deliver a reproducible cell number to each row in a plate and/or a series of plates, as well as the ability to achieve an even distribution of cells throughout a given well. The following procedure assures that cells are reproducibly transferred from flask to microtiter plates, and cells are evenly distributed across the surface of each well.

[0044] The first step in preparing the microtiter plates is, of course, preparing and monitoring the monolayer as described above. The following protocol is exemplary and susceptible of variation as will be apparent to one skilled in the art. Cells are removed from the culture flask and a cell pellet is prepared by centrifugation. The cell pellet derived from the monolayer is then suspended in 5 ml of the growth medium and mixed in a conical tube with a vortex for 6 to 10 seconds. The tube is then rocked back and forth 10 times. A 36 .mu.l droplet from the center of the conical tube is pipetted onto one well of a 96 well plate. A fresh pipette is then used to pipette a 36 .mu.l aliquot of trypan blue solution, which is added to the same well, and the two droplets are mixed with repeated pipette aspiration. The resulting admixture is then divided between two hemocytometer chambers for examination using a standard light microscope. Cells are counted in two out of four hemocytometer quadrants, under 10.times. magnification. Only those cells which have not taken up the trypan blue dye are counted. This process is repeated for the second counting chamber. An average cell count per chamber is thus determined. Using means known in the art, the quadrant count values are checked, logged, multiplied by 10.sup.4 to give cells/ml, and the total amount of fluid (growth medium) necessary to suspend remaining cell aliquots is calculated accordingly.

[0045] After the desired concentration of cells in medium has been determined, additional cell aliquots from the monolayer are suspended in growth medium via vortex and rocking and loaded into a Terasaki dispenser known in the art. Aliquots of the prepared cell suspension are delivered into the microtiter plates using Terasaki dispenser techniques known in the art. A plurality of plates may be prepared from a single cell suspension as needed. Plates are then wrapped in sterile wet cotton gauze and incubated in an incubator box by means known in the art.

[0046] After the microtiter plates have been prepared, exposure of the cells therein to active agent and/or radiation is conducted according to the following exemplary protocol. During this portion of the inventive assay, the appropriate amount of specific active agent is transferred into the microtiter plates prepared as described above. A general protocol, which may be adapted, follows. Each microtiter plate is unwrapped from its wet cotton gauze sponge and microscopically examined for cell adhesion. Control solution is dispensed into delineated rows of wells within the grid in the microtiter plate, and appropriate aliquots of active agent to be tested are added to the remaining wells in the remaining rows. Ordinarily, sequentially increasing concentrations of the active agent or higher doses of

radiation being tested are administered into progressively higher numbered rows in the plate. The plates are then rewrapped in their gauze and incubated in an incubator box at 37.degree. C. under 5% CO.sub.2. After a predefined exposure time, the plates are unwrapped, blotted with sterile gauze to remove the agent, washed with Hank's Balance Salt Solution, flooded with growth medium, and replaced in the incubator in an incubator box for a predefined time period, after which the plates may be fixed and stained for evaluation.

[0047] Fixing and staining may be conducted according to a number of suitable procedures; the following is representative. After removal of the plates from the incubator box, culture medium is poured off and the plates are flooded with Hank's Balance Salt Solution. After repeated flooding (with agitation each time) the plates are then flooded with reagent grade ethanol for 2-5 minutes. The ethanol is then poured off. Staining is accomplished with approximately 5 ml of Giemsa Stain per plate, although volume is not critical and flooding is the goal. Giemsa stain should be left in place 5 min. .+-.30 seconds as timing influences staining intensity. The Giemsa stain is then poured off and the plates are dipped three times in cold tap water in a beaker. The plates are then inverted, shaken vigorously, and air dried overnight (with plate lids off) on a rack on a laboratory bench. Cells per well are then counted manually or by automated and/or computerized means, to derive data regarding chemosensitivity of cells at various concentrations of exposure. One particularly useful computer operating environment for counting cells is the commercially available OPTIMATE compiler, which is designed to permit an optical counting function well suited to computerized cell counting procedures and subsequent calculations.

[0048] The above procedures do not change appreciably when cell growth promoters are assayed rather than cell arresting agents such as chemotherapeutic or radiotherapeutic agents. The present assay allows cell death or cell growth to be monitored with equal ease. In any case, optimization of use of the present system will involve the comparative testing of a variety of candidate active agents for selection of the best candidate for patient treatment based upon the in vitro results. One particularly advantageous embodiment of the above described invention comprises a two-stage assay for cytotoxicity followed by evaluation of longer-term inhibitory effect. Chemotherapeutic agents may thus be evaluated separately for both their direct chemotherapeutic effect as well as for their longer duration efficacy.

[0049] As discussed in brief, above, in parallel with growth of the prime or reference culture, a control culture can be grown. The control culture is a culture of normal cells taken from the same patient from whom the prime culture is collected. The control culture can derive from an epithelial cell sample or can be collected from the same organ as the prime culture so long as the control culture contains no malignant cells. More than one control culture can be maintained. For instance, cultures of both normal skin cells and normal cells of an organ from which the malignancy is derived can be maintained. The value of maintaining a control culture is many fold. Primarily, the control culture serves as a negative control (or positive control, depending upon the marker to be analyzed) in the various analyses to be carried out on the prime culture, the reference culture or subcultures thereof.

[0050] A second value of the control culture is an indicator of toxicity, the toxicity or undesirable effects of a given therapy upon normal cells. For instance, in the segregated analysis of chemotherapeutic agents described above, concomitant analysis of the same agents on segregated sites of the control culture would yield an indication of cytotoxicity of the agent with regard to malignant cells versus the toxicity of the agent to control cells. A therapeutic index can be calculated based on the ratio of cytotoxicity to malignant cells to toxicity. Cytotoxicity and toxicity can be quantified as a percentage or fraction of cells killed by a given therapy, or as a percentage or fraction of cells surviving a given therapy. A therapeutic index is a ratio of these percentages or fractions and is reflective of the desirability of a given treatment in a patient. An optimal treatment would be maximally cytotoxic (or even cytostatic) to the malignant cells and minimally toxic to a patient's normal cells.

[0051] Other indices may be generated, depending upon the desired effect of a therapy. For instance, if a desired therapy is designed to up-regulate a malignancy-specific antigen to promote destruction of the malignancy by a patient's immune system, an index could be generated to discern a treatment which reflects maximal up-regulation of the antigen in the malignant cells and minimal or negative

up-regulation in a patient's normal cells. A similar index can be calculated based upon down-regulation of a desired marker (i.e., an angiogenic factor) which can be assayed as either a secreted or a cellular marker and reflects maximal down-regulation of the marker with minimal toxicity or other undesirable effects on the control culture.

[0052] Often the diseased cells express a cellular marker that is indicative of a certain disease state or lack thereof. However, one aspect of the culture techniques of the present invention is that the cultured diseased cells do not necessarily have to be the cells expressing the factor to be assayed. One question that inevitably arises when considering whether a serum marker is indicative of a particular cancer cell is, which cells produce the marker, the cell or the tissue in which the cancer cells grow? See e.g. Singhal et al., p 610. By co-culturing the cancerous tissue within a multicellular particulate of its originating tissue, the cells (both the diseased cells or the surrounding cells) are better able to retain their production of characteristic markers.

[0053] Identification of one or more active agents or chemotherapeutic agents is peripheral to the present invention, which is intended for the efficacy screening of any or all of them as to a given patient. Literally any active agent may be screened according to the present invention; listing exemplary active agents is thus omitted here.

[0054] One important focus of the present invention thus includes the simplicity of the present system--cohesive multicellular particulates of the patient tissue to be tested are used to form cell monolayers; growth of those monolayers is monitored for accurate prediction of correlating growth of the same cells in vivo; and differing concentrations of a number of active agents may be tested for the purpose of determining not only the most appropriate agent but the most appropriate concentration of that agent for actual patient exposure (according to the calculated cell growth rates). It is also important to note, in the context of the invention, that the present system allows in vitro tests to be conducted in suspensions of tissue culture monolayers grown in nutrient medium under fast conditions (a matter of weeks), rather than with single cell progeny produced by dilution cloning over long periods of time. In some cases, the present invention is a two stage assay for both cytotoxicity and the longer-term growth inhibitory.

[0055] It is additionally possible to increase the value of the assay with the use of staining compositions and protocols designed to characterize the malignant cells thus grown. In other words, the tissue preparation and cell culturing technique itself offers a first assurance that the cells grown out of the tumor are really the malignant tumor cells and not fibroblasts or other nonmalignant cells of no diagnostic value. As a separate confirmation, the present staining compositions and protocols offer a second, independent assurance that the cells subject to diagnostic or prognostic assay are in fact malignant cells in culture. One important characterization has to do with the nature of the malignant cells as epithelial, which is in turn an indicator of the carcinoma type of malignancy. Other characterizations of malignant cells are intended to fall within the scope of the present invention as well, although the characterization of the cells as epithelial or not is of primary importance.

[0056] The technique is practiced as follows. The same cell culturing and well distribution process is used as in the cytotoxicity assay described above, but rather than exposing the cells to chemotherapeutic or other agents, the cells are instead fixed and stained. With the stain or stain cocktail described below, the epithelial cells are identified by their intermediate filaments and/or specific membrane antigens by means of a monoclonal antibody immunoperoxidase technique. The fixative used can be any fixative which does not alter the cellular molecular markers of interest. The fixed, stained cells are then counted. If the specimen is positive for epithelial cells, the process is complete. If the specimen is negative for epithelial cells, an independent fixing and staining process is subsequently completed, with fresh cells from identical wells, using Vimentin as a stain to confirm the non-epithelial nature of the cells.

[0057] The importance of having a stain or stain cocktail (i.e., antibody cocktail), as well as an overall protocol, for identifying epithelial cells in biopsies of malignant tumors is as follows. In the basic cytotoxicity assay, the tissue culture technique is designed to grow out the cells of the tumor of origin and in fact consistently does so. Despite such reliable predictability, however, the fact that

the cells of the tumor of origin did in fact grow out, and not fibroblasts or other cells, must be confirmed with independent proof before the cells can be used with complete assurance in the appropriate patient assay(s). The present technology provides a means to obtain this confirmation, which in turn furthers the interests of good laboratory and medical practice.

[0058] As a general consideration, the staining compounds or compositions of interest for use in the present technology are those which bind with cellular molecular markers unique either to epithelial or to non-epithelial cells. A further aspect of the invention therefore inheres in the following two aspects: the improvement of the cytotoxicity assay by adding the epithelial staining protocol with any known epithelial stain; and the further improvement wherein specially designed stain cocktails maximize the likelihood that the presence of any known intermediate filament or specific membrane antigen, characteristic of epithelial cells, will be identified if present.

[0059] Many carcinomas are positive for any one of the intermediate filaments or specific membrane antigens characteristic of epithelial cells; virtually all if not all carcinomas are positive for one of a number of such intermediate filaments or specific membrane antigens. For example, "epithelial membrane antigen" (EMA) glycoproteins are known in the art and can be bound with various antiepithelial membrane antigen antibodies including monoclonal antibodies. Cytokeratin is another important epithelial cell marker and binding reagents including monoclonal antibodies are available which are specific to cytokeratin. While antisera can be raised in vivo against markers such as EMA glycoproteins and cytokeratin, as a practical matter commercially available polyclonal or monoclonal antibodies are used in the following protocols, with monoclonal antibodies being preferred.

[0060] Binding of the epithelial marker is revealed with associated staining procedures and reactions which give a visual indication that the marker binding took place. Those skilled in the art already appreciate various techniques already available--in the general field of "immunocytochemistry" --to reveal antibody-antigen reactions. One known way to accomplish this visualization when antibody binding reagents are used is with the "labeled streptavidin procedure". In this procedure, after the specimen is exposed to antibodies specific to the target antigen, a secondary "link" antibody is added. The secondary biotinylated "link" antibody consists of anti-mouse and anti-rabbit antibodies which bind universally to most primary monoclonal or polyclonal antibodies. The "link" will also connect to the tertiary reagent (peroxidase-labeled streptavidin) through chemical bonding between the biotin on the secondary reagent and the streptavidin on the streptavidin/peroxidase conjugate. Staining is completed by incubating the specimen and primary, secondary and tertiary agents in the presence of a chromogen, so that the peroxidase and the chromogen form a visible precipitate. Alternatively, a fluorescein-based detection system can be used to visualize the primary antibody, or a third alternative known in the art as the digoxigenin-conjugated detection system may be used.

[0061] Of the various epithelial markers, three have received the most widespread attention in the literature: EMA glycoproteins, cytokeratin, and carcinoembryonic antigen. In the context of this invention, the first two are the most important because literally any epithelial cell will have at least either one EMA glycoprotein on the surface thereof or a cytokeratin intermediate filament present. Therefore, the present invention resides not only in binding and staining for an epithelial marker on the surfaces of the specimen cells, but in simultaneously assaying for either or both of EMA glycoprotein(s) and cytokeratin. The cocktails of the present invention therefore contain binding reagents for both EMA glycoproteins and cytokeratin and, importantly, are selected to include the most generally applicable binding reagents in combination so that the cocktail has the broadest binding scope possible. The cocktails identified in Examples 1 and 2, for example, represent a combination of two general binding reagents (containing a total of three monoclonal antibodies) for cytokeratin, admixed with a general binding reagent for EMA glycoprotein. The dual benefit of this admixture of general binding agents is that the incidence of false negatives for epithelial cells is minimized, and the visible staining reactions are generally stronger when the combined binding reagents are used in lieu of a single binding reagent.

[0062] Although the binding reagents and other reagents identified in the Examples are the preferred reagents for use in the context of the invention, the invention is intended to encompass epithelial-specific binding and staining reagents generally.

These include, without limitation: Boehringer-Mannheim AE1 anti-cytokeratin antibody; Boehringer-Mannheim AE3 anti-cytokeratin antibody; Boehringer-Mannheim AE1/AE3 anti-cytokeratin antibody (AE1 and AE3 in admixture); Becton-Dickinson CAM 5.2 antibody, DAKO EMA antibody, Biomeda's Anti-Cytokeratin Cocktail CK22, Biomeda's Anti-Cytokeratin Cocktail CK23, Biomeda's Anti-Pan-Cytokeratin CK56, Biomeda's polyclonal goat or rabbit anti-cytokeratin antisera, ScyTek Laboratories' anti-EMA antigen antibody clone E29, and many others. Those skilled in the art and in possession of the guidance provided herein can readily determine alternative, equivalent binding and staining reagents and cocktails, to accomplish the disclosed result. These binding agents and cocktails may be used in combination with any known visualization system, such as the streptavidin, fluorescein- and digoxigenin-conjugated systems identified above.

[0063] As a control, Vimentin antibody is used as a binding alternative either in conjunction with binding and staining of the test cells, or subsequently thereto. In the context of this invention, Vimentin can be considered a binding reagent which is specific to non-epithelial cells of mesenchymal origin.

[0064] In a further aspect of the present invention, immunological markers may be monitored in applications requiring up- or down- regulation of such markers (i.e., Major histocompatibility complex molecules). This aspect of the present invention can be especially useful in transplantation applications where, for instance, through chemical or biological means rejection of transplanted cells is sought to be avoided by down-regulation of the various transplantation antigens present on the cells to be transplanted. The present invention would be especially useful in monitoring such immunoregulation.

[0065] Lastly, cell morphology can be assayed by culturing cells of, i.e., the prime culture or the reference culture, removing the cells from the surface upon which they grow, centrifuging cells into a loose pellet and growing the cell pellet over a defined time period. By growing cells in this manner, it is possible to view the cohesive morphology of cells in a cluster resembling a tumor.

EXAMPLE 1

Radiation Therapy

[0066] Separate 50 mg samples from residual tissue from specimens of three human glioblastomas and one human ovarian carcinoma were minced in medium with sterile scissors to a particle size of roughly 1 mm.sup.3 and with a particle size distribution between about 0.25 and about 1.5 mm.sup.3. The medium was Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin. Each 50 mg sample was minced and was divided into four groups of particulates and each of 16 groups was charged to a separate labeled culture flask containing the above-described medium. Visual confirmation was made that the particulates were evenly distributed along the bottom of each flask and the flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the particulates grew into monolayers.

[0067] Enough cells were then removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets for each of the 16 flasks. Each cell pellet was then suspended in 5 ml of the above-described medium and was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10 times. A 36 ml droplet from the center of each tube was then pipetted into one well of a 96-well microtiter plate together with an equal amount of trypan blue, plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10.times. magnification--only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated and by means known in the art the optimum concentration of cells in the medium was determined.

[0068] Accommodating the above calculations, additional cell aliquots from the 16 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. Cells were plated into 60-well microtiter

plates at a concentration of 100 cells per well.

[0069] Twenty-four (24) hours later, the cells were irradiated using a Siemens Stabilipan X-ray machine at 250 kVp, 15 mA with a dose rate of 75 rad/minute. For each radiation dose from 1Gy to 6Gy, cell number per well was monitored as a function of time through five days post-irradiation.

[0070] Cell number relative to controls was determined and survival curves were fit to the data. The rate of decrease in survival as a function of time was proportional to dose. A differential radiation response among the four cell lines was observed.

EXAMPLE 2

Immuno Therapy

[0071] Separate 50 mg samples from residual tissue from specimens of a human brain tumor, renal carcinoma, and breast carcinoma were minced in medium with sterile scissors to a particle size of roughly 1 mm.sup.3 and with a particle size distribution between about 0.25 and about 1.5 mm.sup.3. The medium was Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin. Each 50 mg sample was minced and was divided into four groups of particulates and each of 12 groups was charged to a separate labeled culture flask containing the above-described medium. Visual confirmation was made that the particulates were evenly distributed along the bottom of each flask and the flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the particulates grew into monolayers.

[0072] Enough cells were then removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets for each of the twelve flasks. Each cell pellet was then suspended in 5 ml of the above-described medium and was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10 times. A 36 ml droplet from the center of each tube was then pipetted into one well a 96-well microtiter plate together with an equal amount of trypan blue, plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10.times. magnification--only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated and by means known in the art the optimum concentration of cells in the medium was determined.

[0073] Accommodating the above calculations, additional cell aliquots from the 12 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. Cells were plated into 60-well microtiter plates at a concentration of 100 cells per well.

[0074] Twenty-four (24) hours post-plating, Activated Natural Killer (ANK) cells were delivered into a row of six wells by means of a micropipette. In each microtiter plate three rows of six wells each served as controls. The effector (ANK cells):target cell (tumor cells) ratio varied from 2.5:1 to 20:1. The ANK cells were exposed to the target cells for four hours. Subsequently, the wells were washed with Hanks Balanced Salt Solution and the number of ANK cells remaining in the wells was observed with a phase contrast microscope. This process was repeated until no ANK cells remained in the wells (usually 3 washes). Following removal of the ANK cells, the tumor cells were incubated in the wells for another 24 hours.

[0075] Cell number relative to control was determined. For the three tumor types increasing the effector: target cell ratio from 2.5:1 to 20:1 resulted in an increase in the number of tumor cells killed by the ANK cells.

EXAMPLE 3

Gene Therapy/Antisense Oligonucleotides

[0076] A 50 mg sample from a residual human mesothelioma was minced in medium with sterile scissors to a particle size of roughly 1 mm.sup.3 and with a particle size

distribution between about 0.25 and about 1.5 mm.sup.3. The medium was Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin. The 50 mg sample was minced and was divided into four groups of particulates and each of four groups was charged to a separate labeled culture flask containing the above-described medium. Visual confirmation was made that the particulates were evenly distributed along the bottom of each flask and the flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the particulates grew into monolayers.

[0077] Enough cells were then removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets for each of the four flasks. Each cell pellet was then suspended in 5 ml of the above-described medium and was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10 times. A 36 ml droplet from the center of each tube was then pipetted into one well of a 96-well microtiter plate together with an equal amount of trypan blue, plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10.times. magnification--only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated and by means known in the art the optimum concentration of cells in the medium was determined.

[0078] Accommodating the above calculations, additional cell aliquots from the 4 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. Cells were plated into 60-well microtiter plates at a concentration of 100 cells per well.

[0079] Twenty-four (24) hours post-plating, antisense oligonucleotide for the urokinase-type plasminogen activator receptor (uPAR) was delivered to wells in the microtiter plate. Proteolysis of plasminogen to plasmin by urokinase-type plasminogen activator has been implicated in the processes of tumor cell proliferation and invasion. The concentrations of the uPAR antisense oligonucleotide were 1, 10 and 100 micromolar. uPAR sense and missense oligonucleotides at the concentrations of 1, 10 and 100 micromolar served as controls. The tumor cells were exposed to the oligonucleotides for 24 hours and then the agents were removed. The cells were allowed to incubate for another 72 hours so that inhibition of cell proliferation could be observed.

[0080] Cell number relative to control was then determined. Antisense oligonucleotides to uPAR suppressed the proliferative activity of the tumor cells in a concentration dependent manner.

EXAMPLE 4

Combination Chemotherapy

[0081] Separate 50 mg samples from residual tissue from specimens from four human ovarian tumors were minced in medium with sterile scissors to a particle size of roughly 1 mm.sup.3 and with a particle size distribution between about 0.25 and about 1.5 mm.sup.3. The medium was Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin. Each 50 mg sample was minced and was divided into 4 groups of particulates and each of 16 groups was charged to a separate labeled culture flask containing the above-described medium. Visual confirmation was made that the particulates were evenly distributed along the bottom of each flask and the flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the particulates grew into monolayers.

[0082] Enough cells were then removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets for each of the 16 flasks. Each cell pellet was then suspended in 5 ml of the above-described medium and was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10 times. A 36 ml droplet from the center of each tube was then pipetted into one well of a 96-well microtiter plate together with an equal amount of trypan blue,

plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10.times. magnification--only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated and by means known in the art the optimum concentration of cells in the medium was determined.

[0083] Accommodating the above calculations, additional cell aliquots from the 16 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. Cells were plated into 60-well microtiter plates at a concentration of 100 cells per well.

[0084] Twenty-four (24) hours post-plating, the chemotherapeutic agent Taxol was applied to the wells in the microtiter plates. The first three treatment rows in the plates (Rows 2, 3, and 4) were designed to have escalating Taxol doses (1.0, 5.0, and 25 .mu.M) with a fixed carboplatin dose (200 .mu.M). The last three treatment rows in the plates (Rows 6, 7, and 9) were designed to have a fixed Taxol dose (5 .mu.M) with an escalating carboplatin dose (50, 200, and 1000 .mu.M). Rows 5 and 9 served as a control. The Taxol exposure time was two hours. Twenty-four hours later, the cells in the wells were exposed to carboplatin for two hours. The tumor cells in the wells were then incubated for another 48 hours.

[0085] Cell number relative to control was determined. For the cells from the four tumor specimens a dose response relationship was observed for both the escalating Taxol/fixed carboplatin and fixed Taxol/escalating carboplatin treatment schema.

EXAMPLE 5

Hormonal Therapy

[0086] Separate 50 mg samples from residual tissue from specimens from four human breast tumors were minced in medium with sterile scissors to a particle size of roughly 1 mm.sup.3 and with a particle size distribution between about 0.25 and about 1.5 mm.sup.3. The medium was Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin. Each 50 mg sample was minced and was divided into four groups of particulates and each of 16 groups was charged to a separate labeled culture flask containing the above-described medium. Visual confirmation was made that the particulates were evenly distributed along the bottom of each flask and the flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the particulates grew into monolayers.

[0087] Enough cells were then removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets for each of the 16 flasks. Each cell pellet was then suspended in 5 ml of the above-described medium and was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10 times. A 36 ml droplet from the center of each tube was then pipetted into one well of a 96-well microtiter plate together with an equal amount of trypan blue, plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10.times. magnification--only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated and by means known in the art the optimum concentration of cells in the medium was determined.

[0088] Accommodating the above calculations, additional cell aliquots from the 16 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. Cells were plated into 60-well microtiter plates at a concentration of 100 cells per well.

[0089] Twenty-four (24) hours post-plating, the antiestrogenic compound Tamoxifen was delivered to wells in the microtiter plates. A stock solution of Tamoxifen was

initially prepared by dissolving 1.5 mg of Tamoxifen powder in 1 ml of absolute ethanol and then adding 9 ml of growth medium. This stock solution was then used to make Tamoxifen solutions in the concentration range of 10 nM to 20 .mu.M. Six doses of Tamoxifen were used for cells from each of the four breast tumor specimens. An ethanol solution at a concentration equivalent to that at the highest Tamoxifen concentration served as a control. The tumor cells were exposed to Tamoxifen for 24 hours and then the agent was removed. The cells were allowed to incubate for another 72 hours so that inhibition of cell proliferation could be observed.

[0090] Cell number relative to control was then determined. There was no effect observed when the ethanol-only control wells were compared to the growth medium-only control wells. The cells of two of the four breast specimens tested showed an inhibition of cell proliferation by Tamoxifen exposure. These responses occurred in the mid to high Tamoxifen concentration ranges.

EXAMPLE 6

Differentiating Agent Therapy ("Biological Response Modification")

[0091] Separate 50 mg samples from residual tissue from specimens from four human breast tumors were minced in medium with sterile scissors to a particle size of roughly 1 mm.sup.3 and with a particle size distribution between about 0.25 and about 1.5 mm.sup.3. The medium was Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin. Each 50 mg sample was minced and was divided into four groups of particulates and each of 16 groups was charged to a separate labeled culture flask containing the above-described medium. Visual confirmation was made that the particulates were evenly distributed along the bottom of each flask and the flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the particulates grew into monolayers.

[0092] Enough cells were then removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets for each of the 16 flasks. Each cell pellet was then suspended in 5 ml of the above-described medium and was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10 times. A 36 ml droplet from the center of each tube was then pipetted into one well of a 96-well microtiter plate together with an equal amount of trypan blue, plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10.times. magnification--only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated and by means known in the art the optimum concentration of cells in the medium was determined.

[0093] Accommodating the above calculations, additional cell aliquots from the 16 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. Cells were plated into 60-well microtiter plates at a concentration of 100 cells per well.

[0094] Twenty-four (24) hours post-plating the differentiating agent retinoic acid was delivered to wells in the microtiter plates. A stock solution of retinoic acid was initially prepared by dissolving retinoic acid powder in 1 ml of dimethyl sulfoxide (DMSO) and then adding 9 ml of growth medium. This stock solution was then used to make retinoic acid solutions in the concentration range of 0.1 to 1.0 mM. Six doses of retinoic acid were used for cells from each of the four breast tumor specimens. A DMSO solution at a concentration equivalent to that at the highest retinoic acid concentration served as a control. The tumor cells were exposed to retinoic acid for 24 hours and then the agent was removed. The cells were allowed to incubate for another 72 hours so that inhibition of cell proliferation could be observed.

[0095] Cell number relative to control was then determined. There was no effect observed when the DMSO-only control wells were compared to the growth medium-only control wells. The cells of three of the four breast specimens tested showed an inhibition of cell proliferation by retinoic acid exposure. These responses occurred

in the mid to high retinoic acid concentration ranges.

EXAMPLE 7

Combined Modality Therapy Drug/Radiation

[0096] Separate 50 mg samples from residual tissue from specimens from two human brain tumors and two human ovarian tumors were minced in medium with sterile scissors to a particle size of roughly 1 mm.sup.3 and with a particle size distribution between about 0.25 and about 1.5 mm.sup.3. The medium was Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin. Each 50 mg sample was minced and was divided into four groups of particulates and each of 16 groups was charged to a separate labeled culture flask containing the above-described medium. Visual confirmation was made that the particulates were evenly distributed along the bottom of each flask and the flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the particulates grew into monolayers.

[0097] Enough cells were then removed from the monolayers grown in the flasks for centrifugation into standard size cell pellets for each of the 16 flasks. Each cell pellet was then suspended in 5 ml of the above-described medium and was mixed in a conical tube with a vortex for 6 to 10 seconds, followed by manual rocking back and forth 10 times. A 36 ml droplet from the center of each tube was then pipetted into one well of a 96-well microtiter plate together with an equal amount of trypan blue, plus stirring. The resulting admixture was then divided between two hemocytometer quadrants for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10.times. magnification--only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was calculated and by means known in the art the optimum concentration of cells in the medium was determined.

[0098] Accommodating the above calculations, additional cell aliquots from the 16 monolayers were separately suspended in growth medium via vortex and rocking and were loaded into a Terasaki dispenser adapted to a 60-well plate. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. Cells were plated into 60-well microtiter plates at a concentration of 100 cells per well.

[0099] Twenty-four (24) hours post-plating, cells in the microtiter plate wells were exposed to the chemotherapeutic agent Taxol. One set of plates was designed to have escalating Taxol doses with (0.5-25.0 .mu.M) with a fixed radiation dose (2Gy). A second set of plates was designed to have a fixed Taxol dose (5 .mu.M) with an escalating radiation dose (1Gy-6Gy). The cells in the plates were irradiated using a Siemans Stabilipan X-ray machine operating at 250 kVp, 15 mA with a dose rate of 75 rad/minute.

[0100] For each of the two treatment schema, cell number per well was monitored as a function of time through 5 days post-treatment. Cell number relative to controls was determined and survival curves were fit. A differential response among the cells from the four tumor specimens was observed. Both additive and synergistic cell killing was noted.

EXAMPLE 8

Initiation of a Prime Culture

[0101] A tumor biopsy of approximately 100 mg of non-necrotic, non-contaminated tissue was harvested from the patient by surgical biopsy and transferred to the laboratory in a standard shipping container. Biopsy sample preparation proceeded as follows. Reagent grade ethanol was used to wipe down the surface of a Laminar flow hood. The tumor was then removed, under sterile conditions, from its shipping container, and cut into quarters with a sterile scalpel. Using sterile forceps, each undivided tissue quarter was then placed in 3 ml sterile growth medium (Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin) and was systematically minced by using two sterile scalpels in a scissor-like motion. The tumor particulates each measured about 1 mm.sup.3. After each tumor quarter was minced, the particles were plated in culture flasks using

- sterile pasteur pipettes (9 explants per T-25 or 20 particulates per T-75 flask). Each flask was then labeled with the patient's code, the date of explanation and any other distinguishing data. The explants were evenly distributed across the bottom surface of the flask, with initial inverted incubation in a 37.degree. C. incubator for 5-10 minutes, followed by addition of about 5-10 ml sterile growth medium and further incubation in the normal, non-inverted position. Flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the explants grew out into a monolayer.

EXAMPLE 9

Unified Tracking System

[0102] a. Growth Rate

[0103] Following initiation of prime cell culture of a tumor specimen, the growth rate of the cells was determined until the chemosensitivity assay was performed. During this time period the growth was monitored by observing the percent of confluency of the cells in a flask. These data provide information valuable as a correlation to possible growth of the tumor in the patient as well as for the interpretation of the results of the chemosensitivity assay.

[0104] Three examples of growth rate data are shown in FIGS. 1A-1C. The percent of confluency of the cultured cells is plotted as a function of time after the initial seeding of the tissue specimen.

[0105] Slow Growth Rate (FIG. 1A): 25% confluent after 19 days

[0106] Moderate Growth Rate (FIG. 1B): 60% confluent after 21 days

[0107] Fast Growth Rate (FIG. 1C): 90% confluent after 11 days

[0108] b. Immunohistochemical Staining for Cell Characterization, etc.

[0109] Many tumor specimens will contain a mixture of cancer and normal cells. Although in many cases tumor cells will readily grow in tissue culture, while the normal cells will not, it is important to be able to distinguish the two cell types. Using immunoperoxidase techniques to stain cells for various intermediate filaments, the differences between normal (fibroblast-like) cells and cells from epithelial tumors were characterized. These techniques can also be used to identify other tumor cell characteristics which may have prognostic value.

[0110] An initial attempt at cultured cell characterization has been to use known epithelial tumor cell lines and a known fibroblast cell line. The epithelial tumor cell lines all have stained positively for a mixture ("cocktail") of epithelial intermediate filament antibodies, (not every line, however, has stained positively for the three antibodies within the mixture [AE1/AE3; Cam 5.2; EMA]). Some of the epithelial tumor cells in culture also stained mildly positive for an antibody against an intermediate filament characteristic of fibroblasts (vimentin). When staining for fibroblast intermediate filament (vimentin) in cell culture, all fibroblast cells were positive. Some focal staining by epithelial tumor cells for vimentin was also present.

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	epithelial cocktail	vimentin
epithelial tumor cells	++	+
fibroblasts	-	++

[0111] Testing of intermediate filaments with antibodies for epithelial cells and vimentin appears to be a method of distinguishing certain characteristics of tumor and normal cells.

[0112] c. Response to Chemotherapy

[0113] The tissue culture chemosensitivity assay has been refined to make it more sensitive for the detection of damage produced by a variety of chemotherapeutic agents. The initial alteration was to allow a 24-hour time period between plating of cells in microtiter wells and the exposure to drugs. This time interval permits cells to be in an active state of proliferation, where they are more sensitive to cell cycle active agents. The second change was to initiate a long-term assay (growth inhibition assay) over a period of about 72 hours. The short-term assay is conducted 24-72 hours after the therapeutic agent is added. The longer time between drug exposure and assay allows for the detection of cell damage which occurs over a protracted period and requires several cell division cycles before it becomes apparent. "CI" is a measure of the relative survival rates of a given cell culture. It is calculated by according to the formula: 2 CI = (1 - No. of cells in treated wells) No. of cells in control wells

[0114] The data for a short-term assay and a long-term assay performed on two sets of patient cultured cells are presented in FIGS. 2A-2F through 5A-5F. The long-term assay (FIGS. 3A-3F and 5A-5F) may both accentuate a positive result obtained from the short-term assay (FIGS. 2A-2F and 4A-4F) and reveal an effect not observed during the short-term assay. The long-term assay is now incorporated into the tissue culture chemosensitivity on a routine basis.

[0115] d. Response to Radiation Therapy

[0116] The use of the microtiter well assay to analyze the direct effect of radiation therapy on tumor cells in culture has resulted in a rapid evaluation method for the determination of inherent cellular radiation response. As an example, two radiation dose-response curves generated from the microtiter well assay are presented in FIGS. 6 and 7. The cells from the tumor specimen in FIG. 6 are more resistant than those of the specimen in FIG. 7. The more resistant tumor has been previously irradiated.

[0117] The microtiter well assay is ideally suited for examination of the interaction of chemotherapeutic agents and radiation. Issues such as the differential sensitivity of drug/radiation combinations and the timing of drug/radiation combinations may be directly addressed with this system. An illustration of chemotherapeutic agent enhancement of radiation response is presented in FIGS. 8A-8C.

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FIG. 8A: Radiation-only at 2 Gy and 4 Gy
FIG. 8B: Taxol 8.5 ng/ml + 2 Gy and 4 Gy
FIG. 8C: Taxol 42.5 ng/ml + 2 Gy and 4 Gy

[0118] e. Response to Cellular Immunotherapy

[0119] Activated lymphocytes are being used as a treatment for some types of cancer. These Activated Natural Killers (ANK) cells have been shown to mediate highly efficient cell killing for some tumor types. The microtiter well assay can be utilized to make a rapid assessment of ANK-induced tumor target cell killing. An illustration of two such interactions is presented in FIGS. 9A and 9B.

[0120] In FIGS. 9A and 9B, the target cells were from a melanoma and a renal carcinoma, respectively. The target cells were exposed to the ANK cells for 4 hours and then the assay was performed. The effector:target cell ratio varied from 1:20 to

1:2.5. The data show increasing cell killing as a function of increasing effector: target ratio.

[0121] f. Use of Tissue Culture Medium for Determination of Factors with Possible Prognostic/Biological Significance

[0122] A number of substances secreted by tumor cells such as Tumor Associated Antigens and Plasminogen Activators and Inhibitors are believed to regulate a variety of processes involved in the progression of malignant disease. Many of these factors are produced by tumor cells growing in tissue culture and are secreted into the growth medium. The measurement of these factors in the medium from cell cultures of tumor specimens may prove to be of predictive value in the assessment of the biological behavior of individual cancers.

[0123] Preliminary work in this area has been on the detection of plasminogen activator inhibitor in the growth medium of glioblastoma cell lines. Plasminogen activator inhibitor expression has been shown to be increased in malignant brain tumors in patients. Medium from glioblastoma cell lines showed an increase in plasminogen activator inhibitor when compared to the medium alone.

[0124] Any or all of the steps of the unified assays and culturing techniques of the present invention may be automated. Indices can be automatically calculated by a computer which is programmed appropriately. Data can be input into the computer either manually or automatically, into a spreadsheet or database program, or the like. The spreadsheet or database program can be programed to reduce the data to the indices described above, or to any other relevant form, i.e., graphical or figurative representations of the data.

[0125] In one example, the cells to be assayed are grown on microtiter plates and assayed for their sensitivity to a chemotherapeutic agent according to the above-described protocols. The microtiter plates are read on an optical scanner and data from the scanner is automatically exported to a computer for calculation of a therapeutic index. Other types of scanners may be utilized depending upon the assay. For instance, a scanner for reading RIA data would be provided if the assay is an RIA assay.

[0126] Although the present invention has been described with respect to specific materials and methods above, the invention is only to be considered limited insofar as is set forth in the accompanying claims.

CLAIMS:

I claim:

1. A method for identifying and monitoring progress of an individual patient having, a malignancy comprising the steps of: a. collecting a specimen of a patient's cells; b. separating the specimen into cohesive multicellular particulates; c. growing a tissue culture monolayer from the multicellular particulates to form a prime culture; and d. monitoring said tissue culture monolayer over a period of time.

2. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 1 further comprising the step of maintaining the prime culture.

3. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 1 further comprising the steps of preparing a reference culture from the prime culture and treating the reference culture with one or more treatments as given to the patient.

4. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 3 further comprising the steps of preparing a subculture of one of the prime culture and the reference culture.

5. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 4 further comprising the step of assaying for a malignancy-specific one of the prime culture, the reference culture, the subculture and tissue culture medium used to grow one of the prime culture, the reference culture or the subculture.

6. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 5 wherein the marker indicates one of aggressiveness and invasiveness of the malignancy.

7. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 5 wherein the marker is indicative of complications associated with the malignancy.

8. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 7 wherein the marker is indicative of a thrombogenic potential.

9. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 5 wherein the marker is identified by one of cytochemistry or immunohistochemistry.

10. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 9 wherein the marker is selected from the group consisting of an estrogen receptor, a progesterone receptor, an oncogene, a product of an oncogene, a marker for multi-drug resistance and a marker for phenotypic characterization.

11. A method for identifying and monitoring progress of a malignancy of an individual patient as claimed in claim 5 wherein one or more of the steps are at least partially automated.

12. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 5 wherein the marker is characterized by a molecular biological technique.

13. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 12 wherein the molecular biological technique characterizes one of tumor cell heterogeneity or specific mutations of cancer-related genes

14. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 2 further comprising the steps of: d. inoculating cells from one of the prime culture, the reference culture or a subculture of the prime culture or of the reference culture into a plurality of segregated sites; and e. treating the plurality of sites with at least one treating means, followed by assessment of sensitivity of cells in the site to the treating means.

15. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 14 wherein one or more of the steps are at least partially automated.

16. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 14 further comprising the step of phenotypically or genotypically analyzing the cells in one or more sites for drug resistance.

17. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 14 further comprising the steps of: f. collecting a specimen of a patient's non-malignant cells; g. separating the non-malignant cells into cohesive multicellular particulates; h. growing a tissue culture monolayer from the multicellular particulates of non-malignant cells to form a control culture; i. inoculating the control culture in a plurality of non-segregated sites; j. treating the plurality of segregated sites of the control culture with the same treating means as the segregated sites of the prime culture or a subculture thereof, followed by assessment of the sensitivity of the segregated cells of the control culture to the treating means; and k. comparing the sensitivity of the segregated cells of the prime culture or a subculture thereof with the sensitivity of the segregated cells of the control culture to the treating means.

18. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 17 wherein the assessment of steps e and j are calculations of the percentage or fraction of cells sensitive to the treatment and further comprising the step of: l. creating a therapeutic index of a ratio of

one of the percentage of or the fraction of sensitive cells or insensitive cells in the segregated cells of the control culture to one of the percentage of or the fraction of sensitive cells or insensitive cells in the segregated cells of the prime culture or subculture thereof.

19. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 18 further comprising the step of programming a computer to automatically perform calculations to create said therapeutic index.

20. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 19 wherein the segregated sites are in a readable plate having a plurality of culture wells and a scanner is used to automatically scan the segregated sites to determine the percentage or fraction of cells sensitive to the treatment and an interface is provided between the scanner and the computer allowing automated input of scanner data into the computer for calculation of the therapeutic index.

21. A method for identifying and monitoring progress of a malignancy in an individual patient as claimed in claim 18 wherein the non-malignant cells are epithelial cells.

22. A method for treating a patient having a malignancy comprising the steps of: a. analyzing a patient's cells prepared according to the method of claim 1 for malignancy-associated markers; b. determining a therapeutic regimen according to the results of the analysis; and c. treating a patient according to the regimen.

23. A method for treating a patient having a malignancy as claimed in claim 22 further comprising the step of treating one of cells cultured as a subculture of the prime culture and cells of the prime culture according to the method of claim 14.

24. A method for treating a patient having a malignancy as claimed in claim 22 wherein the analyzing step further includes the steps of: i. inoculating cells from one of the prime culture, the reference culture or a subculture of the prime culture or the reference culture into a plurality of segregated sites; ii. treating the plurality of sites with at least one treating means, followed by assessment of sensitivity of cells in the site to the treating means; and iii. determining a therapeutic index for each treating means according to the method of claim 18.

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TITLE: STAINING AGENTS AND PROTOCOLS FOR CHARACTERIZING MALIGNANT CELLS IN CULTURE

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ABSTRACT:

An improved system for screening a multiple of candidate therapeutic or chemotherapeutic agents for efficacy as to a specific patient, in which a tissue sample from the patient is harvested, cultured and separately exposed to a plurality of treatments and/or therapeutic agents for the purpose of objectively identifying the best treatment or agent for the particular patient. Specific method innovations such as tissue sample preparation techniques render this method practically as well as theoretically useful. The identity of the malignant cells in culture is advantageously confirmed using binding reagents/staining systems specific for epithelial cells, since carcinomas are ubiquitously epithelial in nature. Cells of interest and thus confirmed as epithelial/carcinomal may then be assayed for sensitive to an infinite variety of malignancy treating agents including chemotherapeutic agents, radiation, immunotherapy, and so on.

FIELD OF THE INVENTION

[0001] The invention relates to screening and testing of active agents, including chemotherapeutic agents, to predict potential efficacy in individual patients in whom treatment with such agents is indicated, and staining compositions and protocols to confirm the identity of the cultured malignant cells of interest.

INTRODUCTION

[0002] All active agents including chemotherapeutic active agents are subjected to rigorous testing as to efficacy and safety prior to approval for medical use in the U.S. Methods of assessing efficacy have included elaborate investigations of large populations in double blind studies as to a given treatment method and/or active agent, with concomitant statistical interpretation of the resulting data, but these conclusions are inevitably generalized as to patient populations taken as a whole.

In many pharmaceutical disciplines and particularly in the area of chemotherapy, however, the results of individual patient therapy may not comport with generalized data--to the detriment of the individual patient. The need has been long recognized for a method of assessing the therapeutic potential of active agents, including but not limited to chemotherapeutic agents, for their efficacy as to a given individual patient, prior to the treatment of that patient.

[0003] Prior art assays already exist which expose malignant tissue of various types to a plurality of active agents, for the purpose of assessing the best choice for therapeutic administration. For example, in Kruczynski, A., et al., "Evidence of a direct relationship between the increase in the in vitro passage number of human non-small-cell-lung cancer primocultures and their chemosensitivity," Anticancer Research, vol. 13, no. 2, pp. 507-513 (1993), chemosensitivity of non-small-cell-lung cancers was investigated in in vivo grafts, in in vitro primocultures and in commercially available long-term cancer cell lines. The increase in chemosensitivity was documented and correlated with morphological changes in the cells in question. Sometimes animal model malignant cells and/or established cell cultures are tested with prospective therapy agents, see, for example, Arnold, J. T., "Evaluation of chemopreventive agents in different mechanistic classes using a rat tracheal epithelial cell culture transformation assay," Cancer Res.., vol. 55, no. 3, pp. 537-543 (1995).

[0004] When actual patient cells are used to form in vitro assays focussed on individual patients, in typical prior art processes the cells are harvested (biopsied) and trypsinized (connective tissue digested with the enzyme trypsin) to yield a cell suspension suitable for conversion to the desired tissue culture form. The in vitro tissue culture cell collections which result from these techniques are generally plagued by their inability accurately to imitate the chemosensitivity of the original tumor or other cell biopsy. Standard cloning and tissue culture techniques are moreover excessively complicated and expensive for use in a patient-by-patient assay setting. A need thus remains for a technique of tissue culture preparation which provides cell cultures, for drug screening purposes, in which after simple preparation the cell cultures react in a manner equivalent to their in vivo reactivity, to enable drug or chemotherapeutic agent screening as to a particular patient for whom such screening is indicated.

SUMMARY OF THE INVENTION

[0005] In order to meet this need, the present invention is an improved system for screening a multiple of candidate therapeutic or chemotherapeutic agents for efficacy as to a specific patient and for confirming the identity of the malignant cells being tested, in which a tissue sample from the patient is harvested, cultured and separately exposed to a plurality of treatments and/or therapeutic agents for the purpose of objectively identifying the best treatment for the cultured cells obtained from the patient. Specific method innovations such as tissue sample preparation techniques render this method practically as well as theoretically useful. One particularly important tissue sample preparation technique is the initial preparation of cohesive multicellular particulates of the tissue sample, rather than enzymatically dissociated cell suspensions or preparations, for initial tissue culture monolayer preparation. With respect to the culturing of malignant cells, for example, it is believed (without any intention of being bound by the theory) that by maintaining the malignant cells within a multicellular particulate of the originating tissue, growth of the malignant cells themselves is facilitated versus the overgrowth of fibroblasts or other cells which tends to occur when suspended tumor cells are grown in culture. Practical monolayers of cells may thus be formed to enable meaningful screening of a plurality of treatments and/or agents. Growth of cells is monitored to ascertain the time to initiate the assay and to determine the growth rate of the cultured cells; sequence and timing of drug addition is also monitored and optimized. By subjecting uniform samples of cells to a wide variety of active agents (and concentrations thereof), the most efficacious agent can be determined. For assays concerning cancer treatment, a two-stage evaluation is contemplated in which both acute cytotoxic and longer term inhibitory effect of a given anti-cancer agent are investigated.

[0006] As an important part of the above technique, staining compositions and protocols are used (1) to identify whether the malignant cells grown in culture are epithelial cells and, if not, (2) to confirm that the cells grown in culture are specifically non-epithelial. The overall method, including the method of characterizing the cells grown in culture with these staining compositions and

protocols as well as unique antibody cocktails therefor, forms the core of the subject matter of this continuation-in-part specification.

DETAILED DESCRIPTION OF THE INVENTION

[0007] The present invention is a system for screening a multiple of candidate therapeutic or chemotherapeutic agents for efficacy as to a specific patient, in which a tissue sample from the patient is harvested and separately exposed to a plurality of treatments and/or therapeutic agents for the purpose of objectively identifying the best treatment or agent. Specific method innovations such as tissue sample preparation techniques render this method practically as well as theoretically useful. One particularly important tissue sample preparation technique is the initial preparation of cohesive multicellular particulates (explants) of the tissue sample, rather than enzymatically dissociated cell suspensions or preparations, for initial tissue culture monolayer preparation. Cell growth, and sequence and timing of drug addition, are monitored and optimized.

[0008] As an important part of the above technique, staining compositions and protocols are used (1) to identify whether the malignant cells grown in culture are epithelial cells and, if not, (2) to confirm that the cells grown in culture are specifically non-epithelial. The overall method, including the method of characterizing the cells grown in culture with these staining compositions and protocols as well as unique antibody cocktails therefor, forms the core of the subject matter of this continuation-in-part specification.

[0009] An important application of the present invention is the screening of chemotherapeutic agents and other antineoplastic therapies against tissue culture preparations of malignant cells from the patients from whom malignant samples are biopsied. Related anti-cancer therapies which can be screened using the inventive system are both radiation therapy and agents which enhance the cytotoxicity of radiation, as well as immunotherapeutic anti-cancer agents. Screening processes for treatments or therapeutic agents for nonmalignant syndromes are also embraced within this invention, however, and include without limitation agents which combat hyperproliferative syndromes, such as psoriasis, or wound healing agents. Nor is the present efficacy assay limited only to the screening of active agents which speed up (healing) or slow down (anti-cancer, anti-hyperproliferative) cell growth because agents intended to enhance or to subdue intracellular biochemical functions may be tested in the present tissue culture system also. For example, the formation or blocking of enzymes, neurotransmitters and other biochemicals may be screened with the present assay methods prior to treatment of the patient.

[0010] When the patient is to be treated for the presence of tumor, in the preferred embodiment of the present invention a tumor biopsy of >100 mg of non-necrotic, non-contaminated tissue is harvested from the patient by any suitable biopsy or surgical procedure known in the art. Biopsy sample preparation generally proceeds as follows under a Laminar Flow Hood which should be turned on at least 20 minutes before use. Reagent grade ethanol is used to wipe down the surface of the hood prior to beginning the sample preparation. The tumor is then removed, under sterile conditions, from the shipping container and is minced with sterile scissors. If the specimen arrives already minced, the individual tumor pieces should be divided into four groups. Using sterile forceps, each undivided tissue quarter is then placed in 3 ml sterile growth medium (Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin) and systematically minced by using two sterile scalpels in a scissor-like motion, or mechanically equivalent manual or automated opposing incisor blades. This cross-cutting motion is important because the technique creates smooth cut edges on the resulting tumor multicellular particulates. Preferably but not necessarily, the tumor particulates each measure 1 mm.sup.3. After each tumor quarter has been minced, the particles are plated in culture flasks using sterile pasteur pipettes (9 explants per T-25 or 20 particulates per T-75 flask). Each flask is then labeled with the patient's code, the date of explanation and any other distinguishing data. The explants should be evenly distributed across the bottom surface of the flask, with initial inverted incubation in a 37.degree. C. incubator for 5-10 minutes, followed by addition of about 5-10 ml sterile growth medium and further incubation in the normal, non-inverted position. Flasks are placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks should be checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the explants will foster growth of cells into a monolayer. With respect to the culturing of malignant cells, it is believed (without any intention of being bound by the theory)

that by maintaining the malignant cells within a multicellular particulate of the originating tissue, growth of the malignant cells themselves is facilitated versus the overgrowth of fibroblasts (or other unwanted cells) which tends to occur when suspended tumor cells are grown in culture.

[0011] The use of the above procedure to form a cell monolayer culture maximizes the growth of malignant cells (and only malignant cells) from the tissue sample, and thus optimizes ensuing tissue culture assay of chemotherapeutic action of various agents to be tested. Enhanced growth of actual malignant cells is only one aspect of the present invention, however; another important feature is the growth rate monitoring system used to oversee growth of the monolayer once formed. Once a primary culture and its derived secondary monolayer tissue culture has been initiated, the growth of the cells is monitored to ascertain the time to initiate the chemotherapy assay and to determine the growth rate of the cultured cells.

[0012] Monitoring of the growth of cells is conducted by counting the cells in the monolayer on a periodic basis, without killing or staining the cells and without removing any cells from the culture flask. The counting may be done visually or by automated methods, either with or without the use of estimating techniques known in the art (counting in a representative area of a grid multiplied by number of grid areas, for example). Data from periodic counting is then used to determine growth rates which may or may not be considered parallel to growth rates of the same cells in vivo in the patient. If growth rate cycles can be documented, for example, then dosing of certain active agents can be customized for the patient. The same growth rate can be used to evaluate radiation treatment periodicity, as well. It should be noted that with the growth rate determinations conducted while the monolayers grow in their flasks, the present method requires no hemocytometry, flow cytometry or use of microscope slides and staining, with all their concomitant labor and cost.

[0013] Protocols for monolayer growth rate generally use a phase-contrast inverted microscope to examine culture flasks incubated in a 37.degree. C. (5% CO.sub.2) incubator. When the flask is placed under the phase-contrast inverted microscope, ten fields (areas on a grid inherent to the flask) are examined using the 10x objective, with the proviso that the ten fields should be non-contiguous, or significantly removed from one another, so that the ten fields are a representative sampling of the whole flask. Percentage cell occupancy for each field examined is noted, and averaging of these percentages then provides an estimate of overall percent confluency in the cell culture. When patient samples have been divided between two or among three or more flasks, an average cell count for the total patient sample should be calculated. The calculated average percent confluency should be entered into a process log to enable compilation of data--and plotting of growth curves--over time. Monolayer cultures may be photographed to document cell morphology and culture growth patterns. The applicable formula is: 1 Percent confluency = estimate of the area occupied by cells total area in an observed field

[0014] As an example, therefore, if the estimate of area occupied by the cells is 30% and the total area of the field is 100%, percent confluency is {fraction (30/100)}, or 30.

[0015] Adaptation of the above protocol for non-tumor cells is straightforward and generally constitutes an equivalent procedure.

[0016] Active agent screening using the cultured cells does not proceed in the initial incubation flask, but generally proceeds using plates such as microtiter plates. The performance of the chemosensitivity assay used for screening purposes depends on the ability to deliver a reproducible cell number to each row in a plate and/or a series of plates, as well as the ability to achieve an even distribution of cells throughout a given well. The following procedure assures that cells are reproducibly transferred from flask to microtiter plates, and cells are evenly distributed across the surface of each well.

[0017] The first step in preparing the microtiter plates is, of course, preparing and monitoring the monolayer as described above. The following protocol is exemplary and susceptible of variation as will be apparent to one skilled in the art. Cells are removed from the culture flask and a cell pellet is prepared by centrifugation. The cell pellet derived from the monolayer is then suspended in 5ml of the growth medium and mixed in a conical tube with a vortex for 6 to 10 seconds. The tube is then rocked back and forth 10 times. A 36.mu.l droplet from the center of the conical tube is pipetted onto one well of a 96 well plate. A fresh pipette is then

used to pipette a 36 .mu.l aliquot of trypan blue solution, which is added to the same well, and the two droplets are mixed with repeated pipette aspiration. The resulting admixture is then divided between two hemocytometer chambers for examination using a standard light microscope. Cells are counted in two out of four hemocytometer quadrants, under 10x magnification. Only those cells which have not taken up the trypan blue dye are counted. This process is repeated for the second counting chamber. An average cell count per chamber is thus determined. Using means known in the art, the quadrant count values are checked, logged, multiplied by 10.sup.4to give cells/ml, and the total amount of fluid (growth medium) necessary to suspend remaining cell aliquots is calculated accordingly.

[0018] After the desired concentration of cells in medium has been determined, additional cell aliquots from the monolayer are suspended in growth medium via vortex and rocking and loaded into a Terasaki dispenser known in the art. Aliquots of the prepared cell suspension are delivered into the microtiter plates using Terasaki dispenser techniques known in the art. A plurality of plates may be prepared from a single cell suspension as needed. Plates are then wrapped in sterile wet cotton gauze and incubated in an incubator box by means known in the art.

[0019] After the microtiter plates have been prepared, exposure of the cells therein to active agent is conducted according to the following exemplary protocol. During this portion of the inventive assay, the appropriate amount of specific active agent is transferred into the microtiter plates prepared as described above. A general protocol, which may be adapted, follows. Each microtiter plate is unwrapped from its wet cotton gauze sponge and microscopically examined for cell adhesion. Control solution is dispensed into delineated rows of wells within the grid in the microtiter plate, and appropriate aliquots of active agent to be tested are added to the remaining wells in the remaining rows. Ordinarily, sequentially increasing concentrations of the active agent being tested are administered into progressively higher numbered rows in the plate. The plates are then rewrapped in their gauze and incubated in an incubator box at 37.degree. C. under 5% CO.sub.2. After a predefined exposure time, the plates are unwrapped, blotted with sterile gauze to remove the agent, washed with Hank's Balance Salt Solution, flooded with growth medium, and replaced in the incubator in an incubator box for a predefined time period, after which the plates may be fixed and stained for evaluation.

[0020] Fixing and staining may be conducted according to a number of suitable procedures; the following is representative. After removal of the plates from the incubator box, culture medium is poured off and the plates are flooded with Hank's Balance Salt Solution. After repeated flooding (with agitation each time) the plates are then flooded with reagent grade ethanol for 2-5 minutes. The ethanol is then poured off. Staining is accomplished with approximately 5 ml of Giemsa Stain per plate, although volume is not critical and flooding is the goal. Giemsa stain should be left in place 5 min..+-.30 seconds as timing influences staining intensity. The Giemsa stain is then poured off and the plates are dipped 3 times in cold tap water in a beaker. The plates are then inverted, shaken vigorously, and air dried overnight (with plate lids off) on a rack on a laboratory bench. Cells per well are then counted manually or by automated and/or computerized means, to derive data regarding chemosensitivity of cells at various concentrations of exposure. One particularly useful computer operating environment for counting cells is the commercially available OPTIMATE compiler, which is designed to permit an optical counting function well suited to computerized cell counting procedures and subsequent calculations.

[0021] The above procedures do not change appreciably when cell growth promoters are assayed rather than cell arresting agents such as chemotherapeutic agents. The present assay allows cell death or cell growth to be monitored with equal ease. In any case, optimization of use of the present system will involve the comparative testing of a variety of candidate active agents, for selection of the best candidate for patient treatment based upon the in vitro results. One particularly advantageous embodiment of the above described invention comprises a two-stage assay for cytotoxicity followed by evaluation of longer-term inhibitory effect. Chemotherapeutic agents may thus be evaluated separately for both their direct chemotherapeutic effect as well as for their longer duration efficacy.

[0022] Identification of one or more active agents or chemotherapeutic agents is peripheral to the present invention, which is intended for the efficacy screening of any or all of them as to a given patient. Literally any active agent may be screened according to the present invention; listing exemplary active agents is thus omitted

here.

[0023] The essence of the invention thus includes the important feature of the simplicity of the present system. Cohesive multicellular particulates of the patient tissue to be tested are used to form cell monolayers. Growth of those monolayers is in turn monitored for accurate prediction of correlating growth of the same cells in vivo. Finally, differing concentrations of a number of active agents may be tested for the purpose of determining not only the most appropriate agent but the most appropriate concentration of that agent for actual patient exposure (according to the calculated cell growth rates) . It is also important to note, in the context of the invention, that the present system allows in vitro tests to be conducted in suspensions of tissue culture monolayers grown in nutrient medium under fast conditions (a matter of weeks), rather than with single cell progeny produced by dilution cloning over long periods of time. In some cases, the present invention is a two stage assay for both cytotoxicity and the longer-term growth inhibitory.

[0024] It has now been determined that, despite the reliability of the above-disclosed technique to grow out only the cells of interest (malignant cells), it is additionally possible to increase the value of the associated assay with the use of staining compositions and protocols designed to characterize the malignant cells thus grown. In other words, the tissue preparation and cell culturing technique itself offers a first assurance that the cells grown out of the tumor are really the malignant tumor cells and not fibroblasts or other nonmalignant cells of no diagnostic value. As a separate confirmation, the present staining compositions and protocols offer a second, independent assurance that the cells subject to diagnostic or prognostic assay are in fact malignant cells in culture. One important characterization has to do with the nature of the malignant cells as epithelial, which is in turn an indicator of the carcinoma type of malignancy. Other characterizations of malignant cells are intended to fall within the scope of the present invention as well, although the characterization of the cells as epithelial or not is of primary importance.

[0025] The technique is practiced as follows. The same cell culturing and well distribution process is used as in the cytotoxicity assay described above, but rather than exposing the cells to chemotherapeutic or other agents, the cells are instead fixed and stained. With the stain or stain cocktail described below, the epithelial cells are identified by their intermediate filaments and/or specific membrane antigens by means of a monoclonal antibody immunoperoxidase technique. The fixative used can be any fixative which does not alter the cellular molecular markers of interest. The fixed, stained cells are then counted. If the specimen is positive for epithelial cells, the process is complete. If the specimen is negative for epithelial cells, an independent fixing and staining process is subsequently completed, with fresh cells from identical wells, using Vimentin as a stain to confirm the non-epithelial nature of the cells.

[0026] The importance of having a stain or stain cocktail (i.e., antibody cocktail), as well as an overall protocol, for identifying epithelial cells in biopsies of malignant tumors is as follows. In the basic cytotoxicity assay, the tissue culture technique is designed to grow out the cells of the tumor of origin and in fact consistently does so. Despite such reliable predictability, however, the fact that the cells of the tumor of origin did in fact grow out, and not fibroblasts or other cells, must be confirmed with independent proof before the cells can be used with complete assurance in the appropriate patient assay(s). The present technology provides a means to obtain this confirmation, which in turn furthers the interests of good laboratory and medical practice.

[0027] As a general consideration, the staining compounds or compositions of interest for use in the present technology are those which bind with cellular molecular markers unique either to epithelial or to non-epithelial cells. The invention inheres in the following two aspects: the improvement of the cytotoxicity assay by adding the epithelial staining protocol with any known epithelial stain; and the further improvement wherein specially designed stain cocktails maximize the likelihood that the presence of any known intermediate filament or specific membrane antigen, characteristic of epithelial cells, will be identified if present.

[0028] Many carcinomas are positive for any one of the intermediate filaments or specific membrane antigens characteristic of epithelial cells; virtually all if not all carcinomas are positive for one of a number of such intermediate filaments or specific membrane antigens. For example, "epithelial membrane antigen" ("EMA")

glycoproteins are known in the art and can be bound with various antiepithelial membrane antigen antibodies including monoclonal antibodies. Cytokeratin is another important epithelial cell marker and binding reagents including monoclonal antibodies are available which are specific to cytokeratin. While antisera can be raised in vivo against markers such as EMA glycoproteins and cytokeratin, as a practical matter commercially available polyclonal or monoclonal antibodies are used in the following protocols, with monoclonal antibodies being preferred.

[0029] Binding of the epithelial marker is revealed with associated staining procedures and reactions which give a visual indication that the marker binding took place. Those skilled in the art already appreciate various techniques already available--in the general field of "immunocytochemistry"--to reveal antibody-antigen reactions. One known way to accomplish this visualization when antibody binding reagents are used is with the "labeled streptavidin procedure". In this procedure, after the specimen is exposed to antibodies specific to the target antigen, a secondary "link" antibody is added. The secondary biotinylated "link" antibody consists of anti-mouse and anti-rabbit antibodies which bind universally to most primary monoclonal or polyclonal antibodies. The "link" will also connect to the tertiary reagent (peroxidase-labeled streptavidin) through chemical bonding between the biotin on the secondary reagent and the streptavidin on the streptavidin/peroxidase conjugate. Staining is completed by incubating the specimen and primary, secondary and tertiary agents in the presence of a chromagen, so that the peroxidase and the chromagen form a visible precipitate. Alternatively, a fluorescein-based detection system can be used to visualize the primary antibody, or a third alternative known in the art as the digoxigenin-conjugated detection system may be used.

[0030] Of the various epithelial markers, three have received the most widespread attention in the literature: EMA glycoproteins, cytokeratin, and carcinoembryonic antigen. In the context of this invention, the first two are the most important because literally any epithelial cell will have at least either one EMA glycoprotein on the surface thereof or a cytokeratin intermediate filament present. Therefore, the present invention resides not only in binding and staining for an epithelial marker on the surfaces of the specimen cells, but in simultaneously assaying for either or both of EMA glycoprotein(s) and cytokeratin. The cocktails of the present invention therefore contain binding reagents for both EMA glycoproteins and cytokeratin and, importantly, are selected to include the most generally applicable binding reagents in combination so that the cocktail has the broadest binding scope possible. The cocktails identified in Examples 1 and 2, for example, represent a combination of two general binding reagents (containing a total of three monoclonal antibodies) for cytokeratin, admixed with a general binding reagent for EMA glycoprotein. The dual benefit of this admixture of general binding agents is that the incidence of false negatives for epithelial cells is minimized, and the visible staining reactions are generally stronger when the combined binding reagents are used in lieu of a single binding reagent.

[0031] Although the binding reagents and other reagents identified in the Examples are the preferred reagents for use in the context of the invention, the invention is intended to encompass epithelial-specific binding and staining reagents generally. These include, without limitation: Boehringer-Mannheim AE1 anti-cytokeratin antibody; Boehringer-Mannheim AE3 anti-cytokeratin antibody; Boehringer-Mannheim AE1/AE3 anti-cytokeratin antibody (AE1 and AE3 in admixture); Becton-Dickinson CAM 5.2 antibody, DAKO EMA antibody, Biomeda's Anti-Cytokeratin Cocktail CK22, Biomeda's Anti-Cytokeratin Cocktail CK23, Biomeda's Anti-Pan-Cytokeratin CK56, Biomeda's polyclonal goat or rabbit anti-cytokeratin antisera, ScyTek Laboratories' anti-EMA antigen antibody clone E29, and many others. Those skilled in the art and in possession of the guidance provided herein can readily determine alternative, equivalent binding and staining reagents and cocktails, to accomplish the disclosed result. These binding agents and cocktails may be used in combination with any known visualization system, such as the streptavidin, fluorescein- and digoxigenin-conjugated systems identified above.

[0032] As a control, Vimentin antibody is used as a binding alternative either in conjunction with binding and staining of the test cells, or subsequently thereto. In the context of this invention, Vimentin can be considered a binding reagent which is specific to non-epithelial cells.

[0033] The following examples are illustrative.

Example 1

[0034] A tumor biopsy of approximately 100 mg of non-necrotic, non-contaminated tissue was harvested from the patient by surgical biopsy and transferred to the laboratory in a standard shipping container. Biopsy sample preparation proceeded as follows. Reagent grade ethanol was used to wipe down the surface of a Laminar flow hood. The tumor was then removed, under sterile conditions, from its shipping container, and cut into quarters with a sterile scalpel. Using sterile forceps, each undivided tissue quarter was then placed in 3 ml sterile growth medium (Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin) and was systematically minced by using two sterile scalpels in a scissor-like motion. The tumor particulates each measured about 1 mm.sup.3. After each tumor quarter was minced, the particles were plated in culture flasks using sterile pasteur pipettes (9 explants per T-25 or 20 particulates per T-75 flask). Each flask was then labeled with the patient's code, the date of explanation and any other distinguishing data. The explants were evenly distributed across the bottom surface of the flask, with initial inverted incubation in a 37.degree. C. incubator for 5-10 minutes, followed by addition of about 5-10ml sterile growth medium and further incubation in the normal, non-inverted position. Flasks were placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks were checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5ml of growth medium, the explants grew out into a monolayer.

[0035] The cells were subsequently removed from the culture flask and a cell pellet was prepared by centrifugation. The cell pellet derived from the monolayer was then suspended in 5ml of the growth medium and mixed in a conical tube with a vortex for 6 to 10 seconds. The tube was then rocked back and forth 10 times. A 36 .mu.l droplet from the center of the conical tube was pipetted onto one well of a 96 well plate. A fresh pipette was then used to pipette a 36 .mu.l aliquot of trypan blue solution, which was added to the same well, and the two droplets were mixed with repeated pipette aspiration. The resulting admixture was then divided between two hemocytometer chambers for examination using a standard light microscope. Cells were counted in two out of four hemocytometer quadrants, under 10x magnification. Only those cells which had not taken up the trypan blue dye were counted. This process was repeated for the second counting chamber. An average cell count per chamber was thus determined. Using means known in the art, the quadrant count values were checked, logged, multiplied by 10.sup.4 to give cells/ml, and the total amount of fluid (growth medium) necessary to suspend remaining cell aliquots was calculated accordingly.

[0036] After the desired concentration of cells in medium was determined, additional cell aliquots from the monolayer were suspended in growth medium via vortex and rocking, and were loaded into a Terasaki dispenser known in the art. Aliquots of the prepared cell suspension were delivered into the microtiter plates using Terasaki dispenser techniques known in the art. A plurality of plates were prepared from a single cell suspension. Plates were then wrapped in sterile wet cotton gauze and incubated in an incubator box overnight, after which the microtiter wells were examined to assure that the cells had settled onto and adhered to each well base.

[0037] One microtiter plate was selected and segregated from the others intended for cytotoxicity assay, and the cells were examined as follows. Each well was overlaid with an aliquot of 95% ethanol for five minutes, 70% ethanol for five minutes, and 30% hydrogen peroxide/70% methanol for thirty minutes. Each well was then rinsed in Tris-saline several times.

[0038] Saving the first and last rows in the multi-well plate as controls, the remaining wells were each inoculated with approximately 8 microliters of an antibody cocktail including 80 microliters CAM 5.2 antibody (Becton-Dickinson), 10 microliters of a 1:30 dilution of AE1/AE3 (Boehringer-Mannheim) and 10 microliters of a 1:10 dilution of EMA antibody (DAKO). The wells were incubated at room temperature for one hour and were then rinsed in several changes of Tris-saline. Using an LSAB (streptavidin) kit, about 8 microliters of the secondary reagent (in the DAKO LSAB2 kit, the secondary reagent is yellow) was added to each well, followed by a one hour room-temperature incubation and several rinses in Tris-saline. The tertiary reagent (pink) was then inoculated in approximately 8 microliter amounts to each well, followed by a one hour room-temperature incubation followed by rinsing. An overlayer of Tris-saline was retained in each well while fresh filtered diaminobenzidine tetrahydrochloride (DAB)/H2O2 solution was prepared. The Tris-saline was discarded from each well and replaced with DAB solution. After a

five minute room-temperature incubation, the cells were rinsed in distilled water, stained in hematoxylin for between 30 seconds and 1 minute, rinsed in warm running tap water for 2-5 minutes and rinsed in distilled water.

[0039] In the rows saved for use as a control, the above protocol was repeated except that the antibody cocktail was replaced with 8 microliters 1:200 dilution Vimentin antibody, with every other step being the same.

[0040] For the test wells, a strong brown stain upon visual inspection confirmed the identity of the cells as epithelial cells. Consistent with this observation, all the wells in the control rows were negative.

Example 2

[0041] Example 1 was repeated except that in the test wells an alternate cocktail of binding reagents was used containing 60 microliters CAM 5.2, 20 microliters of a 1:30 dilution of AE1/AE3, and 20 microliters of a 1:10 dilution of EMA antibody. The test wells all stained positive (brown) for epithelial cells; the control wells were negative for non-epithelial cells.

[0042] Although the present invention has been described with respect to specific materials and methods above, the invention is only to be considered limited insofar as is set forth in the accompanying claims. For example, although solid tumors have been discussed above, any malignant cells including but not limited to blood, lymph and other cells may be subjected to the present protocols.

CLAIMS:

We claim:

1. A method for assessing sensitivity of patient cells comprising the steps of: a) obtaining a malignant tissue specimen; b) separating said specimen into multicellular particulates; c) growing a tissue culture monolayer from said cohesive multicellular particulates; d) inoculating cells from said monolayer into a plurality of segregated sites; e) binding and staining some of said plurality of sites with a staining protocol including at least a staining protocol including one binding agent specific for epithelial cells; f) treating the remainder of said plurality of sites with at least one agent; g) examining said plurality of sites; and h) assessing sensitivity of the cells in said remainder of said plurality of sites.

2. The method according to claim 1 wherein said plurality of segregated sites comprise a plate containing a plurality of wells therein.

3. The method according to claim 2 wherein said binding agent specific for epithelial cells includes at least one of anti-cytokeratin antibody and anti-epithelial-membrane-antigen antibody.

4. The method according to claim 3 wherein said binding agent specific for epithelial cells includes both anti-cytokeratin antibody and anti-epithelial-membrane-antigen antibody and further wherein the binding resulting from the addition of the binding agent is made visible via a chemical reaction with a streptavidin/peroxidase conjugate and a chromagen.

5. An antibody cocktail containing at least two monoclonal antibodies specific to cytokeratin and at least one monoclonal antibody specific to epithelial membrane antigen.

6. An antibody cocktail containing 80 microliters CAM 5.2 antibody (Becton-Dickinson), 10 microliters of a 1:30 dilution of AE1/AE3 (Boehringer-Mannheim) and 10 microliters of a 1:10 dilution of EMA antibody (DAKO).

7. An antibody cocktail containing 60 microliters CAM 5.2 antibody (Becton-Dickinson), 20 microliters of a 1:30 dilution of AE1/AE3 (Boehringer-Mannheim), and 20 microliters of a 1:10 dilution of EMA antibody (DAKO).

8. A method of confirming the epithelial character of malignant cells grown in culture, for use in a subsequent sensitivity assay, comprising binding a sample of said malignant cells with at least one binding agent specific for epithelial cell

markers, conducting a chemical reaction with said binding reagent to render visible any resultant binding and to confirm the epithelial character of the cells, and subjecting fresh samples of the same malignant cells to sensitivity assays in vitro.

9. A method of confirming the epithelial character of malignant cells grown in culture, for use in a subsequent sensitivity assay, comprising binding a sample of said malignant cells with at least one binding agent further comprising an antibody cocktail containing at least two anti-cytokeratin monoclonal antibodies and at least one anti-epithelial membrane antigen monoclonal antibody, conducting a chemical reaction with said binding reagent to render visible any resultant binding, and subjecting fresh samples of the same malignant cells to sensitivity assays in vitro.

10. A method of confirming the epithelial character of malignant cells grown in culture, for use in a subsequent sensitivity assay, comprising binding a sample of said malignant cells with at least one binding agent further comprising an antibody cocktail containing 80 microliters CAM 5.2 antibody (Becton-Dickinson), 10 microliters of a 1:30 dilution of AE1/AE3 (Boehringer-Mannheim) and 10 microliters of a 1:10 dilution of EMA antibody (DAKO), conducting a chemical reaction with said binding reagent to render visible any resultant binding, and subjecting fresh samples of the same malignant cells to sensitivity assays in vitro.

11. A method of confirming the epithelial character of malignant cells grown in culture, for use in a subsequent sensitivity assay, comprising binding a sample of said malignant cells with at least one binding agent further comprising an antibody cocktail containing 60 microliters CAM 5.2 antibody (Becton-Dickinson), 20 microliters of a 1:30 dilution of AE1/AE3 (Boehringer-Mannheim), and 20 microliters of a 1:10 dilution of EMA antibody (DAKO), conducting a chemical reaction with said binding reagent to render visible any resultant binding, and subjecting fresh samples of the same malignant cells to sensitivity assays in vitro.

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ABSTRACT:

An improved system for screening a multiple of candidate therapeutic or chemotherapeutic agents for efficacy as to a specific patient, in which a tissue sample from the patient is harvested, cultured and separately exposed to a plurality of treatments and/or therapeutic agents for the purpose of objectively identifying the best treatment or agent for the particular patient. Specific method innovations such as tissue sample preparation techniques render this method practically as well as theoretically useful. One particularly important tissue sample preparation technique is the initial preparation of cohesive multicellular particulates of the tissue sample, rather than enzymatically dissociated cell suspensions or preparations, for initial tissue culture monolayer preparation. With respect to the culturing of malignant cells, for example, it is believed (without any intention of being bound by the theory) that by maintaining the malignant cells within a multicellular particulate of the originating tissue, growth of the malignant cells themselves is facilitated versus the overgrowth of fibroblasts or other cells which tends to occur when suspended tumor cells are grown in culture. Practical monolayers of cells may thus be formed to enable meaningful screening of a plurality of treatments and/or agents. Growth of cells is monitored to ascertain the time to initiate the assay and to determine the growth rate of the cultured cells; sequence and timing of drug addition is also monitored and optimized. By subjecting uniform samples of cells to a wide variety of active agents (and concentrations thereof), the most promising agent and concentration for treatment of a particular patient can be determined. For assays concerning cancer treatment, a two-stage evaluation is contemplated in which both acute cytotoxic and longer term inhibitory effect of a

given anti-cancer agent are investigated.

FIELD OF THE INVENTION

[0001] The invention relates to screening and testing of active agents, including chemotherapeutic agents, to predict potential efficacy in individual patients in whom treatment with such agents is indicated.

INTRODUCTION

[0002] All active agents including chemotherapeutic active agents are subjected to rigorous testing as to efficacy and safety prior to approval for medical use in the United States. Methods of assessing efficacy have included elaborate investigations of large populations in double blind studies as to a given treatment method and/or active agent, with concommitant statistical interpretation of the resulting data, but these conclusions are inevitably generalized as to patient populations taken as a whole. In many pharmaceutical disciplines and particularly in the area of chemotherapy, however, the results of individual patient therapy may not comport with generalized data--to the detriment of the individual patient. The need has been long recognized for a method of assessing the therapeutic potential of active agents, including but not limited to chemotherapeutic agents, for their efficacy as to a given individual patient, prior to the treatment of that patient.

[0003] Prior art assays already exist which expose malignant tissue of various types to a plurality of active agents, for the purpose of assessing the best choice for therapeutic administration. For example, in Kruczynski, A., et al., "Evidence of a direct relationship between the increase in the in vitro passage number of human non-small-cell-lung cancer primocultures and their chemosensitivity," Anticancer Research, vol. 13, no. 2, pp. 507-513 (1993), chemosensitivity of non-small-cell-lung cancers was investigated in in vivo grafts, in in vitro primocultures and in commercially available long-term cancer cell lines. The increase in chemosensitivity was documented and correlated with morphological changes in the cells in question. Sometimes animal model malignant cells and/or established cell cultures are tested with prospective therapy agents, see for example Arnold, J. T., "Evaluation of chemopreventive agents in different mechanistic classes using a rat tracheal epithelial cell culture transformation assay," Cancer Res., vol. 55, no. 3, pp. 537-543 (1995).

[0004] When actual patient cells are used to form in vitro assays focussed on individual patients, in typical prior art processes the cells are harvested (biopsied) and trypsinized (connective tissue digested with the enzyme trypsin) to yield a cell suspension suitable for conversion to the desired tissue culture form. The in vitro tissue culture cell collections which result from these techniques are generally plagued by their inability accurately to imitate the chemosensitivity of the original tumor or other cell biopsy. Standard cloning and tissue culture techniques are moreover excessively complicated and expensive for use in a patient-by-patient assay setting. A need thus remains for a technique of tissue culture preparation which provides cell cultures, for drug screening purposes, in which after simple preparation the cell cultures react in a manner equivalent to their in vivo reactivity, to enable drug or chemotherapeutic agent screening as to a particular patient for whom such screening is indicated.

SUMMARY OF THE INVENTION

[0005] In order to meet this need, the present invention is an improved system for screening a multiple of candidate therapeutic or chemotherapeutic agents for efficacy as to a specific patient, in which a tissue sample from the patient is harvested, cultured and separately exposed to a plurality of treatments and/or therapeutic agents for the purpose of objectively identifying the best treatment for the cultured cells obtained from the patient. Specific method innovations such as tissue sample preparation techniques render this method practically as well as theoretically useful. One particularly important tissue sample preparation technique is the initial preparation of cohesive multicellular particulates of the tissue sample, rather than enzymatically dissociated cell suspensions or preparations, for initial tissue culture monolayer preparation. With respect to the culturing of malignant cells, for example, it is believed (without any intention of being bound by the theory) that by maintaining the malignant cells within a multicellular particulate of the originating tissue, growth of the malignant cells themselves is facilitated versus the overgrowth of fibroblasts or other cells which tends to occur

when suspended tumor cells are grown in culture. Practical monolayers of cells may thus be formed to enable meaningful screening of a plurality of treatments and/or agents. Growth of cells is monitored to ascertain the time to initiate the assay and to determine the growth rate of the cultured cells; sequence and timing of drug addition is also monitored and optimized. By subjecting uniform samples of cells to a wide variety of active agents (and concentrations thereof), the most efficacious agent can be determined. For assays concerning cancer treatment, a two-stage evaluation is contemplated in which both acute cytotoxic and longer term inhibitory effect of a given anti-cancer agent are investigated.

DETAILED DESCRIPTION OF THE INVENTION

[0006] The present invention is a system for screening a multiple of candidate therapeutic or chemotherapeutic agents for efficacy as to a specific patient, in which a tissue sample from the patient is harvested and separately exposed to a plurality of treatments and/or therapeutic agents for the purpose of objectively identifying the best treatment or agent. Specific method innovations such as tissue sample preparation techniques render this method practically as well as theoretically useful. One particularly important tissue sample preparation technique is the initial preparation of cohesive multicellular particulates (explants) of the tissue sample, rather than enzymatically dissociated cell suspensions or preparations, for initial tissue culture monolayer preparation. Cell growth, and sequence and timing of drug addition, are monitored and optimized.

[0007] An important application of the present invention is the screening of chemotherapeutic agents and other antineoplastic therapies against tissue culture preparations of malignant cells from the patients from whom malignant samples are biopsied. Related anti-cancer therapies which can be screened using the inventive system are both radiation therapy and agents which enhance the cytotoxicity of radiation, as well as immunotherapeutic anti-cancer agents. Screening processes for treatments or therapeutic agents for nonmalignant syndromes are also embraced within this invention, however, and include without limitation agents which combat hyperproliferative syndromes, such as psoriasis, or wound healing agents. Nor is the present efficacy assay limited only to the screening of active agents which speed up (healing) or slow down (anti-cancer, anti-hyperproliferative) cell growth because agents intended to enhance or to subdue intracellular biochemical functions may be tested in the present tissue culture system also. For example, the formation or blocking of enzymes, neurotransmitters and other biochemicals may be screened with the present assay methods prior to treatment of the patient.

[0008] When the patient is to be treated for the presence of tumor, in the preferred embodiment of the present invention a tumor biopsy of>100 mg of non-necrotic, non-contaminated tissue is harvested from the patient by any suitable biopsy or surgical procedure known in the art. Biopsy sample preparation generally proceeds as follows under a Laminar Flow Hood which should be turned on at least 20 minutes before use. Reagent grade ethanol is used to wipe down the surface of the hood prior to beginning the sample preparation. The tumor is then removed, under sterile conditions, from the shipping container and is minced with sterile scissors. If the specimen arrives already minced, the individual tumor pieces should be divided into four groups. Using sterile forceps, each undivided tissue quarter is then placed in 3 ml sterile growth medium (Standard F-10 medium containing 17% calf serum and a standard amount of Penicillin and Streptomycin) and systematically minced by using two sterile scalpels in a scissor-like motion, or mechanically equivalent manual or automated opposing incisor blades. This cross-cutting motion is important because the technique creates smooth cut edges on the resulting tumor multicellular particulates. Preferably but not necessarily, the tumor particulates each measure 1 mm.sup.3. After each tumor quarter has been minced, the particles are plated in culture flasks using sterile pasteur pipettes (9 explants per T-25 or 20 particulates per T-75 flask). Each flask is then labelled with the patient's code, the date of explantation and any other distinguishing data. The explants should be evenly distributed across the bottom surface of the flask, with initial inverted incubation in a 37.degree. C. incubator for 5-10 minutes, followed by addition of about 5-10 ml sterile growth medium and further incubation in the normal, non-inverted position. Flasks are placed in a 35.degree. C., non-CO.sub.2 incubator. Flasks should be checked daily for growth and contamination. Over a period of a few weeks, with weekly removal and replacement of 5 ml of growth medium, the explants will foster growth of cells into a monolayer. With respect to the culturing of malignant cells, it is believed (without any intention of being bound by the theory) that by maintaining the malignant cells within a multicellular particulate of the

originating tissue, growth of the malignant cells themselves is facilitated versus the overgrowth of fibroblasts (or other unwanted cells) which tends to occur when suspended tumor cells are grown in culture.

[0009] The use of the above procedure to form a cell monolayer culture maximizes the growth of malignant cells from the tissue sample, and thus optimizes ensuing tissue culture assay of chemotherapeutic action of various agents to be tested. Enhanced growth of actual malignant cells is only one aspect of the present invention, however; another important feature is the growth rate monitoring system used to oversee growth of the monolayer once formed. Once a primary culture and its derived secondary monolayer tissue culture has been initiated, the growth of the cells is monitored to ascertain the time to initiate the chemotherapy assay and to determine the growth rate of the cultured cells.

[0010] Monitoring of the growth of cells is conducted by counting the cells in the monolayer on a periodic basis, without killing or staining the cells and without removing any cells from the culture flask. The counting may be done visually or by automated methods, either with or without the use of estimating techniques known in the art (counting in a representative area of a grid multiplied by number of grid areas, for example). Data from periodic counting is then used to determine growth rates which may or may not be considered parallel to growth rates of the same cells in vivo in the patient. If growth rate cycles can be documented, for example, then dosing of certain active agents can be customized for the patient. The same growth rate can be used to evaluate radiation treatment periodicity, as well. It should be noted that with the growth rate determinations conducted while the monolayers grow in their flasks, the present method requires no hemocytometry, flow cytometry or use of microscope slides and staining, with all their concommitant labor and cost.

[0011] Protocols for monolayer growth rate generally use a phase-contrast inverted microscope to examine culture flasks incubated in a 37.degree. C. (5% CO.sub.2) incubator. When the flask is placed under the phase-contrast inverted microscope, ten fields (areas on a grid inherent to the flask) are examined using the 10.times. objective, with the proviso that the ten fields should be non-contiguous, or significantly removed from one another, so that the ten fields are a representative sampling of the whole flask. Percentage cell occupancy for each field examined is noted, and averaging of these percentages then provides an estimate of overall percent confluency in the cell culture. When patient samples have been divided between two or among three or more flasks, an average cell count for the total patient sample should be calculated. The calculated average percent confluency should be entered into a process log to enable compilation of data--and plotting of growth curves--over time. Monolayer cultures may be photographed to document cell morphology and culture growth patterns. The applicable formula is: 1 Percent confluency = estimate of the area occupied by cells total area in an observed field

[0012] As an example, therefore, if the estimate of area occupied by the cells is 30% and the total area of the field is 100%, percent confluency is 30/100, or 30.

[0013] Adaptation of the above protocol for non-tumor cells is straightforward and generally constitutes an equivalent procedure.

[0014] Active agent screening using the cultured cells does not proceed in the initial incubation flask, but generally proceeds using plates such as microtiter plates. The performance of the chemosensitivity assay used for screening purposes depends on the ability to deliver a reproducible cell number to each row in a plate and/or a series of plates, as well as the ability to achieve an even distribution of cells throughout a given well. The following procedure assures that cells are reproducibly transferred from flask to microtiter plates, and cells are evenly distributed across the surface of each well.

[0015] The first step in preparing the microtiter plates is, of course, preparing and monitoring the monolayer as described above. The following protocol is exemplary and susceptible of variation as will be apparent to one skilled in the art. Cells are removed from the culture flask and a cell pellet is prepared by centrifugation. The cell pellet derived from the monolayer is then suspended in 5 ml of the growth medium and mixed in a conical tube with a vortex for 6 to 10 seconds. The tube is then rocked back and forth 10 times. A 36 .mu.l droplet from the center of the conical tube is pipetted onto one well of a 96 well plate. A fresh pipette is then used to pipette a 36 .mu.l aliquot of trypan blue solution, which is added to the same well, and the two droplets are mixed with repeated pipette aspiration. The

resulting admixture is then divided between two hemocytometer chambers for examination using a standard light microscope. Cells are counted in two out of four hemocytometer quadrants, under 10.times. magnification. Only those cells which have not taken up the trypan blue dye are counted. This process is repeated for the second counting chamber. An average cell count per chamber is thus determined. Using means known in the art, the quadrant count values are checked, logged, multiplied by 10.sup.4 to give cells/ml, and the total amount of fluid (growth medium) necessary to suspend remaining cell aliquots is calculated accordingly.

[0016] After the desired concentration of cells in medium has been determined, additional cell aliquots from the monolayer are suspended in growth medium via vortex and rocking and loaded into a Terasaki dispenser known in the art. Aliquots of the prepared cell suspension are delivered into the microtiter plates using Terasaki dispenser techniques known in the art. A plurality of plates may be prepared from a single cell suspension as needed. Plates are then wrapped in sterile wet cotton gauze and incubated in an incubator box by means known in the art.

[0017] After the microtiter plates have been prepared, exposure of the cells therein to active agent is conducted, according to the following exemplary protocol. During this portion of the inventive assay, the appropriate amount of specific active agent is tranferred into the microtiter plates prepared as described above. A general protocol, which may be adapted, follows. Each microtiter plate is unwrapped from its wet cotton gauze sponge and microscopically examined for cell adhesion. Control solution is dispensed into delineated rows of wells within the grid in the microtiter plate, and appropriate aliquots of active agent to be tested are added to the remaining wells in the remaining rows. Ordinarily, sequentially increasing concentrations of the active agent being tested are administered into progressively higher numbered rows in the plate. The plates are then rewrapped in their gauze and incubated in an incubator box at 37.degree. C. under 5% CO.sub.2. After a predefined exposure time, the plates are unwrapped, blotted with sterile gauze to remove the agent, washed with Hank's Balance Salt Solution, flooded with growth medium, and replaced in the incubator in an incubator box for a predefined time period, after which the plates may be fixed and stained for evaluation.

[0018] Fixing and staining may be conducted according to a number of suitable procedures; the following is representative. After removal of the plates from the incubator box, culture medium is poured off and the plates are flooded with Hank's Balance Salt Solution. After repeated flooding (with agitation each time) the plates are then flooded with reagent grade ethanol for 2-5 minutes. The ethanol is then poured off. Staining is accomplished with approximately 5 ml of Giemsa Stain per plate, although volume is not critical and flooding is the goal. Giemsa stain should be left in place 5 min..+-.30 seconds as timing influences staining intensity. The Giemsa stain is then poured off and the plates are dipped 3 times cold tap water in a beaker. The plates are then inverted, shaken vigorously, and air dried overnight (with plate lids off) on a rack on a laboratory bench. Cells per well are then counted manually or by automated and/or computerized means, to derive data regarding chemosensitivity of cells at various concentrations of exposure. One particularly useful computer operating environment for counting cells is the commercially available OPTIMATE compiler, which is designed to permit an optical counting function well suited to computerized cell counting procedures and subsequent calculations.

[0019] The above procedures do not change appreciably when cell growth promoters are assayed rather than cell arresting agents such as chemotherapeutic agents. The present assay allows cell death or cell growth to be monitored with equal ease. In any case, optimization of use of the present system will involve the comparative testing of a variety of candidate active agents, for selection of the best candidate for patient treatment based upon the in vitro results. One particularly advantageous embodiment of the above described invention comprises a two-stage assay for cytotoxicity followed by evaluation of longer-term inhibitory effect. Chemotherapeutic agents may thus be evaluated separately for both their direct chemotherapeutic effect as well as for their longer duration efficacy.

[0020] Identification of one or more active agents or chemotherapeutic agents is peripheral to the present invention, which is intended for the efficacy screening of any or all of them as to a given patient. Literally any active agent may be screened according to the present invention; listing exemplary active agents is thus omitted here.

[0021] The essence of the invention thus includes the important feature of the simplicity of the present system--cohesive multicellular particulates of the patient tissue to be tested are used to form cell monolayers; growth of those monolayers is monitored for accurate prediction of correlating growth of the same cells in vivo; and differing concentrations of a number of active agents may be tested for the purpose of determining not only the most appropriate agent but the most appropriate concentration of that agent for actual patient exposure (according to the calculated cell growth rates). It is also important to note, in the context of the invention, that the present system allows in vitro tests to be conducted in suspensions of tissue culture monolayers grown nutrient medium under fast conditions (a matter of weeks), rather than with single cell progeny produced by dilution cloning over long periods of time. In some cases, the present invention is a two stage assay for both cytotoxicity and the longer-term growth inhibitory.

[0022] Although the present invention has been described with respect to specific materials and methods above, the invention is only to be considered limited insofar as is set forth in the accompanying claims.

CLAIMS:

I claim:

1. A method for assessing chemosensitivity of patient cells comprising the steps of: a) harvesting a specimen of a patient's tissue, cells ascites, or effusion fluid; b) separating said specimen into multicellular particulates; c) growing a tissue culture monolayer from said cohesive multicellular particulates; d) inoculating cells from said monolayer into a plurality of segregated sites; and e) treating said plurality of sites with at least one active agent, followed by assessment of chemosensitivity of the cells in said site to at least one active agent.

2. The method according to claim 1 wherein step a) further comprises the step of a) preparing a specimen which was harvested from a sample of patient tumor tissue;.

3. The method according to claim 1 wherein said plurality of segregated sites further comprises a plate containing a plurality of wells therein.

4. The method according to claim 1 wherein step e) further comprises the step of: e) treating said plurality of sites with a plurality of active agents at varied concentrations, followed by assessment of optimal chemosensitivity with respect to a single active agent at a single concentration.

5. The method according to claim 1 wherein step e) further comprises the step of: e) treating said plurality of sites with a plurality of active agents over a length of time adequate to permit assessment of both initial cytotoxic effect and longer-term inhibitory effect of at least one of said plurality of active agents.

6. The method according to claim 1 wherein the chemosensitivity assayed according to step e) is anti-cancer sensitivity.

7. The method according to claim 1 wherein step d) is accomplished using a Terasaki dispenser.

8. The method according to claim 1 wherein the cells in step d) are prepared in suspension prior to inoculation into a plurality of wells in a culture plate.

9. The method according to claim 1, wherein said active agent is a chemotherapeutic agent.

10. The method according to claim 1, wherein said active agent is a wound healing agent.

11. The method according to claim 1, wherein said active agent is a radiation therapy and/or a radiation therapy sensitizing or ameliorating agent.

12. The method according to claim 1, where said active agent is an immunotherapeutic agent.